METHODS FOR USING PRIMERS THAT ENCODE ONE STRAND OF A DOUBLE-STRANDED PROMOTER

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to U.S. Provisional Patent Application Serial No. 60/428,013, filed November 21, 2002. The entire disclosure of all priority applications is specifically incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] Not Applicable.

FIELD OF THE INVENTION

[0003] The present invention relates generally to methods, compositions and kits for using an RNA polymerase for making transcription products corresponding to a target sequence by obtaining circular single-stranded DNA transcription substrates using a promoter primer that encodes one strand of a double-stranded promoter. The invention has broad applicability for research, diagnostic and therapeutic applications, such as preparing cDNA corresponding to mRNA, making sense or anti-sense probes, detecting gene- or organism-specific sequences, or making RNAi.

BACKGROUND OF THE INVENTION

Description of Related Art

[0004] It is well known in the art that a target sequence can be transcribed *in vitro* or *in vivo* if the target sequence is inserted into a cloning vector downstream of a transcription promoter, such as, but not limited to a T7 RNA polymerase promoter or another T7-type RNA polymerase promoter and the resulting clone is incubated under transcription conditions with an RNA polymerase that recognizes the respective promoter or the clone is transformed into a host cell that can express the RNA polymerase (e.g., see Studier, FW et al., pp. 60-89 in Methods in Enzymology, Vol. 185, ed. by Goeddel, DV, Academic Press, 1990, incorporated herein by reference). Under suitable conditions, this system can also be used to express a protein encoded by a target sequence comprising a gene *in vitro* or, in an appropriate host cell, *in vivo*.

[0005] There are many reasons for which it is beneficial to transcribe one or more target nucleic acid sequences. For example, *in vitro* transcription is frequently used in methods to make probes corresponding to mRNA sequences in samples in order to profile the expression of genes in cells of a particular type versus another type or versus a similar type in response to different conditions or stimuli. Contemporary gene expression profiling is typically performed by simultaneously hybridizing labeled probes prepared from one or more samples to arrays or microarrays having sequences for up to hundreds or thousands of different genes attached to a surface. In some cases, the expression or lack of expression of particular genes may correlate with or be indicative of the presence or status of a disease state, such as, but not limited to, a cancer.

[0006] A number of the methods for making probes corresponding to a target sequence for such purposes are known in the art. Examples of methods that involve *in vitro* transcription for making probes for gene expression profiling are described in: Murakawa et al., DNA 7:287-295, 1988; Phillips and Eberwine, Methods in Enzymol. Suppl. 10:283-288, 1996; Ginsberg et al., Ann. Neurol. 45:174-181, 1999; Ginsberg et al., Ann. Neurol. 48:77-87, 2000; VanGelder et al., Proc. Natl. Acad. Sci. USA 87:1663-1667, 1990; Eberwine et al., Proc. Natl. Acad. Sci. USA 89:3010-3014, 1992; U.S. Patent Nos. 5,021,335; 5,168,038; 5,545,522; 5,514,545; 5,716,785; 5,891,636; 5,958,688; 6,291,170; and PCT Patent Applications WO 00/75356 and WO 02/065093.

[0007] Still other methods use *in vitro* transcription as part of a process for amplifying and detecting one or more target nucleic acid sequences in order to detect the presence of a pathogen, such as a viral or microbial pathogen, that is a causative agent for a disease or to detect a gene sequence that is related to a disease or the status of a disease for medical purposes. Examples of methods that use *in vitro* transcription for this purpose include U.S. Patent Nos. 5,130,238; 5,194,370; 5,399,491; 5,409,818; 5,437,990; 5,466,586; 5,554,517; 5,665,545; 6,063,603; 6,090,591; 6,100,024; 6,410,276; Kwoh et al., Proc. Natl. Acad. Sci. USA 86:1173, 1989; Fahy et al, In: PCR Methods and Applications, pp. 25-33, 1991; PCT Patent Application Nos. WO 89/06700 and WO 91/18155; and European Patent Application Nos. 0427073 A2 and 0427074 A2.

[0008] The use of a single-stranded DNA template for transcription is not common. However, Milligan et al. (Nucleic Acids Res., 15: 8783-8798, 1987) showed that a double-stranded DNA template is not necessary to synthesize RNA using T7 RNA polymerase, provided that the single-stranded DNA template contained a double-stranded -17 to +1 T7

promoter region. Also, a method for synthesizing RNA using a single-stranded DNA template that is non-covalently immobilized on a solid support by annealing to a complementary promoter sequence is described in U.S. Pat. No. 5,700,667. In addition, Japanese Patent No. JP4304900 of Aono Toshiya et al. disclose synthesis of RNA using a circular single-stranded template with a double-stranded T7 RNA polymerase promoter. The circular substrate for transcription disclosed in JP4304900 is obtained by ligation of a linear probe having target-complementary 3'- and 5'-end sequences which are adjacent when the linear probe is annealed to a target sequence in the sample.

[0009] Nevertheless, all of the methods referenced above that obtain a probe or a transcription substrate by replication of a target nucleic acid sequence involve synthesis of linear double-stranded DNA comprising a double-stranded transcription promoter that is operably joined to a double-stranded template.

[0010] The reason the methods in the art that obtain a probe or transcription substrate by replication of a target sequence have used double-stranded DNA templates is understood when the processes for these methods are examined in detail. For example, the methods described by Van Gelder et al. in U.S. Patent Nos. 5,545,522; 5,716,785; and 5,891,636 use a promoter primer to synthesize a double-stranded transcription substrate. First, the promoter primer, which comprises a promoter sequence that is 5'-of a 3'-end portion, wherein the 3'-end portion is complementary to one or more mRNA target sequences, is used to reverse transcribe the mRNA target. Then, second-strand cDNA is synthesized using RNA or a hairpin from the first-strand cDNA as a primer. Since RNA polymerase transcribes a template strand in a 5'-to-3' direction, the only way a promoter sequence can be joined to a target sequence in the correct orientation using a promoter primer according to this method is to replicate the promoter sequence of the promoter primer at the 3'-end of the second-strand cDNA. That is, the promoter sequence in this promoter primer directs transcription of RNA using second-strand cDNA as a template. The template strand for transcription therefore has a Watson-Crick base-pairing sequence that is identical to an mRNA target sequence in the sample and the transcription product comprises anti-sense RNA. The single-stranded promoter sequence used in the promoter primer of Van Gelder et al., which is one strand of a functional double-stranded promoter, is referred to herein as an "anti-sense promoter" and the corresponding promoter primer is referred to herein as an "anti-sense promoter primer". Similarly, the promoter sequence of a double-stranded promoter that is operably joined to the template strand is referred to as a "sense promoter

sequence" herein and a promoter primer that comprises this sequence is referred to as a "sense promoter primer."

[0011] The present invention provides, in part, a novel method for using sense promoter primers in order to obtain transcription products corresponding to a target sequence. The method makes transcription substrates comprising a single-stranded DNA template that encodes the target sequence, which ssDNA template is operably joined to a functional double-stranded promoter. Additional aspects of the invention will be understood from the specification below.

BRIEF SUMMARY OF THE INVENTION

[0012] The methods in the art use anti-sense promoter primers, whereas some embodiments of the present invention provide methods that use sense promoter primers. Thus, by way of example, but without limiting the invention with respect to the promoter sequence or the respective RNA polymerase used for transcription, whereas the anti-sense T7 promoter sequence and +1 base used in a promoter primer in U.S. Patent Nos. 5,545,522; 5,716,785; and 5,891,636 is:

[0013] (5' TAATACGACTCACTATAG 3');

[0014] a corresponding sense T7 promoter sequence and +1 base that can be used in a promoter primer in some embodiments of the present invention is:

[0015] (5' CTATAGTGAGTCGTATTA 3').

[0016] The present invention provides methods, compositions and kits for making transcription products corresponding to a target nucleic acid sequence in a sample by transcription of circular transcription substrates. A first step of the method to obtain circular transcription substrates, as shown in Figure 1, is primer extension of sense promoter primers using a target sequence as a template. The resulting sense promoter-containing first-strand cDNA is then ligated using a ligase under ligation conditions to obtain circular sense promoter-containing first-strand cDNA. A circular transcription substrate is obtained by annealing of a complementary anti-sense promoter oligo to the circular sense promoter-containing first-strand cDNA. The circular transcription substrate comprises a double-stranded transcription promoter that is operably joined to a single-stranded template corresponding to a target sequence.

[0017] The invention also comprises methods to obtain linear transcription substrates. In one embodiment, shown in Figure 2, circular sense promoter-containing first-strand cDNA

(obtained as described above) is linearized using methods described herein below. Then, the resulting linear sense promoter-containing first-strand cDNA is then complexed with an anti-sense promoter oligo to obtain a linear transcription substrate. Alternatively, the circular transcription substrate can be linearized to obtain a linear transcription substrate comprising a double-stranded transcription promoter that is operably joined to a single-stranded template. Both circular and linear transcription substrates can be used to make transcription products corresponding to target nucleic acid sequences.

[0018] The invention also includes methods for amplifying the amount of transcription products obtained from transcription of a transcription substrate. One embodiment, shown in Figure 3, uses transcription products obtained from transcription of a first transcription substrate to make additional transcription substrates using a sense promoter primer according to methods of the present invention described herein.

[0019] The invention also comprises embodiments of the methods that obtain a transcription substrate by annealing of an anti-sense promoter oligo to a sense promoter-containing first-strand or second-strand cDNA which use an oligonucleotide comprising an anti-sense promoter oligo that is attached or immobilized on a surface, wherein the sense promoter containing first-strand or second-strand cDNA is complexed with the immobilized anti-sense promoter oligo to obtain an immobilized transcription substrate which is used to obtain transcription products corresponding to the target nucleic acid. Immobilization of the anti-sense promoter oligo permits methods and assays using dipsticks, arrays and the like. Methods for using an immobilized circular or linear transcription substrates to detect transcription products corresponding to a target sequence are shown in Figure 4 and Figure 5, respectively.

[0020] If a transcription substrate is obtained by using a sense promoter primer to prime synthesis of first-strand cDNA and the target nucleic acid as a template, transcription of the transcription substrate results in sense transcription products.

[0021] However, in some embodiments of the invention, anti-sense transcription products are obtained. One method for obtaining anti-sense transcription products is shown in Figure 6. Briefly, this method uses a primer comprising anti-sense promoter sequence to prime synthesis of first-strand cDNA. Then, after circularizing the first-strand cDNA by ligation, a concatemeric second-strand cDNA is obtained by rolling circle replication using a strand displacement primer and a strand-displacing DNA polymerase. The second-strand cDNA comprises sense promoter sequences. Annealing of an anti-sense promoter oligo to

the sense promoter sequences permits transcription of anti-sense transcription products with respect to the target sequence of the target nucleic acid.

[0022] In another embodiment (not illustrated), a sense promoter primer is used according to the invention to obtain transcription substrates for synthesis of anti-sense transcription products. In these embodiments of the invention, synthesis of first-strand cDNA that is complementary to the target nucleic acid comprising the target sequence is carried out using a primer that lacks a promoter sequence. If the sequence of the first-strand cDNA is known, a sense promoter primer that has a sequence at its 3'-end that is complementary to a specific 3' sequence of the first-strand cDNA can then be used to prime synthesis of second -strand cDNA. Alternatively, the first-strand cDNA can be "tailed" and an additional sequence that is not complementary to the target sequence can be added to the 3'-end of the first-strand cDNA using methods, such as those known in the art (e.g., U.S. Patent No. 5,962,272 and Schmidt, WM and Mueller, MW, Nucleic Acids Res., 27: e31, 1999 or as described herein. The additional sequence that is added to the 3'-end of the first-strand cDNA can be used as a unique priming site for annealing of a sense promoter primer of the invention. A tail and/or additional sequence for binding of a sense promoter primer is especially useful if the sequence of the 3'-portion of the first-strand cDNA is not known or if the sense promoter primer is used to prime synthesis of a multiplicity of target sequences comprising the target nucleic acid (e.g., to prime synthesis of first-strand cDNA corresponding to substantially all mRNA molecules in a sample). Primer extension of the sense promoter primer using first-strand cDNA as a template (including the tail or additional sequence, if present) results in synthesis of sense promoter-containing second-strand cDNA, which can be used to obtain a circular sense promoter-containing second-strand cDNA by ligation using a ligase under ligation conditions. Then, a circular transcription substrate of the invention can be obtained by annealing an anti-sense promoter oligo to the circular sense promoter-containing second-strand cDNA. The circular transcription substrate can be linearized using methods described herein to obtain a linear transcription substrate. Alternatively, the circular sense promoter-containing second-strand cDNA can be linearized and then annealed to an anti-sense promoter oligo to obtain a linear transcription substrate. Transcription of the resulting circular or linear transcription substrates yield transcription products that are anti-sense transcription products with respect to the starting target nucleic acid.

[0023] Yet another aspect of the invention is a signaling system for detecting and/or quantifying analytes of any type, including, without limitation, protein, carbohydrate, nucleic acid or other analytes. Embodiments of this aspect of the invention use Signal Probes, including RCT Signal Probes or LINT Signal Probes, which comprise a sense promoter that is joined to the 3'-end of a signal template in order to detect and/or quantify an ABS-oligo comprising an analyte-binding substance that is joined to an oligonucleotide comprising an anti-sense promoter sequence. Transcription of the Signal Probe-ABS-oligo indicates the presence and/or quantity of the analyte in the sample. In this aspect of the invention, the template sequence is not obtained by copying a target sequence in a sample. Rather, the template that is transcribed by an RNA polymerase to detect and/or quantify an analyte serves as a "signal" for the analyte-binding substance and for the analyte. Embodiments of the invention that use Signal Probes enable simple assays and methods, such as, but not limited to those illustrated in Figures 7 and 8..

BRIEF DESCRIPTION OF THE DRAWINGS

[0024] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[0025] FIG. 1--Schematic of an embodiment of the invention that uses a sense promoter primer to obtain a circular transcription substrate.

[0026] FIG. 2-- Schematic of an embodiment of the invention for obtaining a linear transcription substrate by linearization of a circular sense promoter-containing first-strand cDNA and complexing with an anti-sense promoter oligo.

[0027] FIG. 3--Schematic of an embodiment of a method or assay that uses a sense promoter primer having a transcription termination sequence in order to obtain a circular transcription substrate that is used to obtain additional transcription product. The assay can be performed in a stepwise or in a continuous manner.

[0028] FIG. 4--Schematic of an assay that is used to detect a transcription product corresponding to a target sequence in a target nucleic acid in a sample. In this example, the sense promoter-containing first-strand cDNA is annealed to an anti-sense promoter sequence that is immobilized on a solid support, such as a dipstick or a bead. The resulting

immobilized circular transcription substrate is transcribed by rolling circle transcription and the transcription product is detected using a molecular beacon.

[0029] FIG. 5--Schematic of an assay that uses a linear transcription substrate made by linearizing a circular sense promoter-containing first-strand cDNA and complexing with an anti-sense promoter oligo that is immobilized on a solid support to make a transcription product corresponding to a target sequence in a target nucleic. The transcription product can be detected by any of a variety of methods known in the art.

[0030] FIG. 6--Embodiment that uses an anti-sense promoter primer. Primer extension of the promoter primer and ligation of the first-strand cDNA makes a circular anti-sense-promoter-containing first-strand cDNA that serves as a template for rolling circle replication, the product of which is a linear sense promoter-containing second-strand cDNA. Annealing of an anti-sense promoter oligo makes a concatemeric linear transcription substrate that is used to make a transcription product.

[0031] FIG. 7--Schematic of an assay that uses an RCT Signal Probe to detect an analyte using an analyte-binding substance comprising a second antibody to which a polynucleotide comprising an anti-sense promoter sequence is joined. The anti-sense promoter sequence complexes the sense promoter of the RCT Signal Probe and thereby generates a substrate for transcription by an RNA polymerase that recognizes the double-stranded promoter and initiates rolling circle transcription therefrom under transcription conditions. In this example, a molecular beacon is used to detect the transcription product, which indicates the presence of the analyte. If performed with controls of known quantities of analyte, the level of analyte can also be determined.

[0032] FIG. 8--Embodiment of a method that uses a LINT Signal Probe to detect, and under suitable conditions, to quantify an analyte in a sample using an analyte-binding substance comprising a second antibody to which a polynucleotide comprising an anti-sense promoter sequence is joined. In this embodiment The anti-sense promoter sequence complexes the sense promoter of a LINT Signal Probe and thereby generates a substrate for transcription by an RNA polymerase that recognizes the double-stranded promoter and initiates linear run-off transcription therefrom under transcription conditions. The transcription product can be detected by any of a variety of methods known in the art.

DETAILED DESCRIPTION OF THE INVENTION

[0033] The present invention relates to novel methods, compositions, and kits for synthesizing RNA corresponding to one or multiple target nucleic acid sequences in or from a sample. The target sequence can comprise at least a portion of one or more target nucleic acids comprising either RNA or DNA from any source. By way of example, but not of limitation, the target nucleic acid sequences that are transcribed using a method of the invention can comprise first-strand cDNA corresponding to from one to substantially all mRNA molecules in a sample. Alternatively, the target nucleic acid sequence that is transcribed can comprise a specific sequence in genomic DNA of a particular organism. The present invention overcomes deficiencies in the art by providing methods to obtain circular DNA transcription substrates that comprise a double-stranded promoter that is operably joined to a single-stranded template for transcription and provides a unique system to synthesize RNAs of a desired sequence. The circular transcription substrates can be used to synthesize RNA for use as probes for expression studies using arrays or microarrays, for RNase protection studies, for *in situ* hybridization studies, for Southern and Northern blot analysis, for the synthesis of defined RNA:DNA hybrids, for RNA interference, for *in vitro* translation, for microinjection, or for nucleic acid amplification. The present invention also allows for the synthesis of derivatized RNA for these or other applications.

[0034] In general, it is envisioned that the RNA or transcription product probes described herein will be useful both as reagents in solution hybridization for detection of expression of corresponding genes, as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to hybridization with selected probes under desired conditions. The selected conditions will depend on the particular circumstances based on the particular criteria required (depending, for example, on the G+C content, type of target nucleic acid, source of nucleic acid, size of hybridization probe, etc.). Following washing of the hybridized surface to remove non-specifically bound probe molecules, hybridization is detected, or even quantified, by means of the label.

Methods for Making Transcription Products Corresponding to a Target Nucleic Acid Sequence

One aspect of the invention comprises a method for making a transcription product corresponding to a target sequence in a target nucleic acid, the method comprising:

[0035] (a) obtaining a target nucleic acid;

[0036] (b) obtaining a sense promoter primer, the sense promoter primer comprising a 5'-end portion comprising a sense transcription promoter and a 3'-end portion that is complementary to the target;

[0037] (c) annealing the sense promoter primer with the target nucleic acid so as to form a target nucleic acid-sense promoter primer complex;

[0038] (d) contacting the target nucleic acid-sense promoter primer complex with a DNA polymerase under polymerization reaction conditions so as to obtain first-strand cDNA that is complementary to the target sequence;

[0039] (e) obtaining first-strand cDNA;

[0040] (f) ligating the first-strand cDNA under ligation conditions so as to obtain circular sense promoter-containing first-strand cDNA;

[0041] (g) obtaining an anti-sense promoter oligo;

[0042] (h) annealing the anti-sense promoter oligo to the circular sense promoter-containing first-strand cDNA so as to obtain a circular transcription substrate;

[0043] (i) obtaining the circular transcription substrate; and

[0044] (j) contacting the circular transcription substrate with an RNA polymerase under transcription conditions, wherein a transcription product is obtained.

[0045] The invention comprises use of any polymerase that synthesizes a transcription product using a circular transcription substrate that is obtained by circularization of a primer extension product obtained by primer extension on a target sequence of a sense promoter primer, the sense promoter primer comprising a sequence that encodes a sense strand of a transcription promoter in its 5'-end portion and a target-complementary sequence in its 3'-end portion, and wherein the sense promoter is operably or functionally joined or linked to the primer extension product comprising the target sequence by ligating the sense promoter sequence to the 3'-end of the primer extension product and complexing the resulting circular ssDNA with an oligo comprising an anti-sense promoter sequence that

is complementary to the sense promoter sequence therein (unless a single-stranded sense pseudopromoter is used in the promoter primer in place of a sense strand for a double-stranded promoter). Thus, the methods of the invention can use any RNA polymerase for which a suitable sense promoter sequence is known or can be obtained.

[0046] Preferred promoters of the invention comprise promoters for T7-type RNA polymerases. By "T7-type RNAPs" we mean T7, T3, phi, phiIIH, W31, gh1, Y, A1122, SP6 and mitochondrial RNAPs, as well as mutant forms of these RNAPs (Sousa et al., U.S. Patent No. 5,849,546; Padilla, R and Sousa, R, Nucleic Acids Res., 15: e138, 2002; Sousa, R and Mukherjee, S, Prog Nucleic Acid Res Mol Biol., 73: 1-41, 2003), such as, but not limited to T7 RNAP Y639F mutant enzyme, T3 RNAP Y573F mutant enzyme, SP6 RNAP Y631F mutant enzyme, T7 RNAP having altered amino acids at both positions 639 and 784, T3 RNAP having altered amino acids at both positions 573 and 785, or SP6 RNAP having altered amino acids at both positions 631 and 779, or other mutant forms of an RNAP that functions in a method of the invention.

[0047] Sense promoters of the invention also comprise single-stranded pseudopromoters or synthetic promoters that are recognized by an RNA polymerase (RNAP) so as to function in a method of the invention. A "pseudopromoter" or "synthetic promoter" of the present invention can be any single-stranded sequence that is identified and/or selected to be functional as a promoter for *in vitro* transcription by an RNA polymerase that binds the promoter with specificity and functions as a promoter for the RNA polymerase in a transcription reaction. That is, a single-stranded pseudopromoter or synthetic promoter that is recognized by the RNA polymerase is not suitable for aspects of the invention that used Signal Probe, which is incorporated herein by reference. Single-stranded promoters for phage N4 vRNAP can also be used in some embodiments, in which case a wild-type or mutant form of N4 mini-vRNAP is used in the method or kit, both of which are discussed elsewhere herein.

[0048] If a pseudopromoter or synthetic promoter is used as a sense promoter in a method or assay of the invention, then the corresponding RNA polymerase for which the pseudopromoter or synthetic promoter was identified and/or selected is used in the method. By way of example, but not of limitation, a sense promoter comprising a ssDNA pseudopromoter can be obtained as described by Ohmichi et al. (Proc. Natl. Acad. Sci. USA 99:54-59, 2002, incorporated herein by reference) and used in a sense promoter primer of a method or assay of the invention that uses *E. coli* RNAP or a T7-type phage

RNAP. If a single-stranded pseudopromoter or synthetic promoter is used in a sense promoter primer of the invention, a circular sense promoter-containing single-stranded cDNA obtained using a method of the invention comprises a circular transcription substrate without annealing an anti-sense promoter oligo. Therefore, no anti-sense promoter oligo is needed in these embodiments. Single-stranded promoters for phage N4 vRNAP can also be used in some embodiments, in which case a wild-type or mutant form of N4 mini-vRNAP is used in the method or kit, both of which are discussed elsewhere herein, and no anti-sense promoter oligo is needed in those embodiments.

A. Definitions and General Methods

1. Transcription Product

[0049] The term "transcription product" as used herein can comprise RNA or, in view of the ability of certain polymerases of the invention, including, without limitation, a T7 RNAP Y639F mutant enzyme or a T7 RNAP mutant enzyme having altered amino acids at both positions 639 and 784 (Sousa et al., U.S. Patent No. 5,849,546; Padilla, R and Sousa, R, Nucleic Acids Res., 15: e138, 2002; Sousa, R and Mukherjee, S, Prog Nucleic Acid Res Mol Biol., 73: 1-41, 2003), to use base-substituted ribonucleotides, such as 5-allyamino-UTP, or non-canonical nucleotide substrates such as dNTPs or 2'-substituted 2'-deoxyribonucleotides such as, but not limited to 2'-fluoro-, 2'-amino-, 2'-methoxy-, or 2'-azido-substituted 2'-deoxyribonucleotides, a transcription product can comprise, in addition to RNA, DNA or modified DNA, or modified RNA, or a mixture thereof. A transcription product does not necessarily have perfect sequence complementarity or identity to the target sequence. For example, a transcription product can include nucleotide analogs such as deoxyinosine or deoxyuridine, intentional sequence alterations, such as sequence alterations introduced through a primer comprising a sequence that is hybridizable, but not complementary, to the target sequence, and/or sequence errors that occur during transcription.

[0050] Also, for a variety of reasons, a nucleic acid or polynucleotide of the invention may comprise one or more modified nucleic acid bases, sugar moieties, or internucleoside linkages. By way of example, some reasons for using nucleic acids or polynucleotides that contain modified bases, sugar moieties, or internucleoside linkages include, but are not limited to: (1) modification of the T_m; (2) changing the susceptibility of the polynucleotide to one or more nucleases; (3) providing a moiety for attachment of a label; (4) providing a

label or a quencher for a label; or (5) providing a moiety, such as biotin, for attaching to another molecule which is in solution or bound to a surface.

2. Sample/Target/Target Nucleic Acid/Target Sequence

[0051] A "sample" or a "biological sample" according to the present invention is used in its broadest sense. A sample is any specimen that is collected from or is associated with a biological or environmental source, or which comprises or contains biological material, whether in whole or in part, and whether living or dead.

[0052] Biological samples may be plant or animal, including human, fluid (e.g., blood or blood fractions, urine, saliva, sputum, cerebral spinal fluid, pleural fluid, milk, lymph, or semen), swabs (e.g., buccal or cervical swabs), solid (e.g., stool), microbial cultures (e.g., plate or liquid cultures of bacteria, fungi, parasites, protozoans, or viruses), or cells or tissue (e.g., fresh or paraffin-embedded tissue sections, hair follicles, mouse tail snips, leaves, or parts of human, animal, plant, microbial, viral, or other cells, tissues, organs or whole organisms, including subcellular fractions or cell extracts), as well as liquid and solid food and feed products and ingredients such as dairy items, vegetables, meat and meat by-products, and waste. Biological samples may be obtained from all of the various families of domestic plants or animals, as well as wild animals or plants.

[0053] Environmental samples include environmental material such as surface matter, soil, water, air, or industrial samples, as well as samples obtained from food and dairy processing instruments, apparatus, equipment, utensils, disposable and non-disposable items. These examples are not to be construed as limiting the sample types applicable to the present invention.

[0054] In short, a sample comprises a specimen from any source that contains or may contain a naturally occurring target nucleic acid.

[0055] A sample on which the assay method of the invention is carried out can be a raw specimen of biological material, such as serum or other body fluid, tissue culture medium or food material. More typically, the method is carried out on a sample that is a processed specimen, derived from a raw specimen by various treatments to remove materials that would interfere with detection of a target nucleic acid or an amplification product thereof. Methods for processing raw samples to obtain a sample more suitable for the assay methods of the invention are well known in the art.

[0056] An "analyte" means a substance whose presence, concentration or amount in a sample is being determined in an assay. An analyte is sometimes referred to as a "target substance" or a "target molecule" or a "target analyte" of an assay. An analyte may also be referred to more specifically. Embodiments of the present invention pertain to analytes that are naturally occurring nucleic acids, and the analyte may be referred to as a "target nucleic acid" or a "target polynucleotide" or a "target oligonucleotide" or a "target sequence," depending on the particular case. A composition, kit, or method of the invention can be used for an "analyte-specific reagent" to detect a target nucleic acid analyte or another analyte or analyte-binding substance in a sample.

[0057] With respect to the present invention, an analyte is often associated with a biological entity that is present in a sample if and only if the analyte is present. Such biological entities include viroids (analyte is, e.g., a nucleic acid or a segment thereof); viruses (analyte is, e.g., a viral genome, or a segment of viral genome); other microorganisms (analyte is, e.g., a segment of the genome or the RNA of the microorganism); abnormal cells, such as cancer cells (analyte is, e.g., an oncogene); or an abnormal gene (analyte is, e.g., a gene segment which includes the altered bases which render the gene abnormal, or a messenger RNA segment which includes altered bases as a result of having been transcribed from the abnormal gene).

[0058] From the description of analyte, it is apparent that the present invention has widespread applicability, including in applications in which nucleic acid probe hybridization assays are often employed. Thus, among other applications, the invention is useful in diagnosing diseases in plants and animals, including humans; and in testing products, such as food, blood, and tissue cultures, for contaminants.

[0059] A "target" of the present invention is a biological organism or material that is the reason or basis for which a biological assay or a diagnostic assay is performed. By way of example, but not of limitation, an assay of the present invention may be performed to detect a target that is a virus which is indicative of a present disease or a risk of future disease (e.g., HIV which is believed to result in AIDS), or a target that is a gene which is indicative of antibiotic resistance (e.g., an antibiotic resistance gene in an infectious pathogenic bacterium), or a target that is a gene which, if absent, may be indicative of disease (e.g., a deletion in an essential gene). In developing assays according to the present invention, it is important to identify target analytes that yield assay results that are sufficiently specific, accurate, and sensitive to be meaningful related to the presence or condition of the target.

[0060] A target analyte that is a "target polynucleotide" or a "target nucleic acid" comprises at least one nucleic acid molecule or portion of at least one nucleic acid molecule, whether the molecule or molecules is or are DNA, RNA, or both DNA and RNA, and wherein each the molecule has, at least in part, a defined nucleotide sequence. The target polynucleotide may also have at least partial complementarity with other molecules used in an assay, such as, but not limited to, primers, splice template oligos, ligation splint oligos, capture probes or detection probes. The target polynucleotide may be single- or double- stranded. A target polynucleotide of the present invention may be of any length. However, it must comprise a polynucleotide sequence of sufficient sequence specificity and length so as to be useful for its intended purpose. By way of example, but not of limitation, a target nucleic acid that is to be detected using a sequence-complementary detection probe must have a sequence of sufficient sequence specificity and length so as remain hybridized by the detection probe under assay hybridization conditions wherein sequences that are not target polynucleotides are not hybridized. A target polynucleotide having sufficient sequence specificity and length for an assay of the present invention may be identified, using methods known to those skilled in the art, by comparison and analysis of nucleic acid sequences known for a target and for other sequences which may be present in the sample. For example, sequences for nucleic acids of many viruses, bacteria, humans (e.g., for genes and messenger RNA), and many other biological organisms can be searched using public or private databases, and sequence comparisons, folded structures, and hybridization melting temperatures (i.e., T_m's) may be obtained using computer software known to those knowledgeable in the art.

[0061] The terms "source of target nucleic acid" or "source of target polynucleotide" refers to any sample that contains a naturally occurring target nucleic acid, RNA or DNA.

[0062] Thus, a method of the present invention can be carried out on nucleic acid from a variety of sources, including unpurified nucleic acids, or nucleic acids purified using any appropriate method in the art, such as, but not limited to, various "spin" columns, cationic membranes and filters, or salt precipitation techniques, for which a wide variety of products are commercially available (e.g., MasterPure™ DNA & RNA Purification Kits from Epicentre Technologies, Madison, WI, USA). Methods of the present invention can also be carried out on nucleic acids isolated from viroids, viruses or cells of a specimen and deposited onto solid supports as described by Gillespie and Spiegelman (J. Mol. Biol. 12:829-842, 1965), including solid supports on dipsticks and the inside walls of microtiter

plate wells. The method can also be carried out with nucleic acid isolated from specimens and deposited on solid support by "dot" blotting (Kafatos, et al., Nucl. Acids Res. 7:1541-1552, 1979); White, and Bancroft, J. Biol. Chem. 257:8569-8572, 1982); Southern blotting (Southern, E., J. Mol. Biol. 98:503-517, 1975); "northern" blotting (Thomas, Proc. Natl. Acad. Sci. USA 77:5201-5205, 1980); and electroblotting (Stellwag, and Dahlberg, Nucl. Acids Res. 8:299-317, 1980). The method can also be carried out for nucleic acids spotted on membranes, on slides, or on chips as arrays or microarrays, or the method can be carried out to prepare probes for detecting or quantifying nucleic acids present in a sample based on hybridization to nucleic acids spotted or synthesized on one of these surfaces. Nucleic acid of specimens can also be assayed by the method of the present invention applied to water phase hybridization (Britten, and Kohne, Science 161:527-540, 1968) and water/organic interphase hybridizations (Kohne, et al., Biochemistry 16:5329-5341, 1977). Water/organic interphase hybridizations have the advantage of proceeding with very rapid kinetics but are not suitable when an organic phase-soluble linking moiety, such as biotin, is joined to the nucleic acid affinity molecule.

[0063] The methods of the present invention can also be carried out on amplification products obtained by amplification of a naturally occurring target nucleic acid, provided that the target sequence in the target nucleic acid is amplified by the method used only if the target nucleic acid is present in the sample. Suitable amplification methods include, but are not limited to, PCR, RT-PCR, NASBA, TMA, 3SR, LCR, LLA, SDA (e.g., Walker et al., Nucleic Acids Res. 20:1691-1696, 1992), RCA, Multiple Displacement Amplification (Molecular Staging), ICAN™ or UCAN™ (TAKARA), Loop-AMP (EIKEN), and SPIA™ or Ribo-SPIA™ (NuGEN Technologies). There are various reasons for using a nucleic acid that is a product of another amplification method as a target nucleic acid for an assay of the present invention, such as, but not limited to, for obtaining more sensitive detection of targets, greater specificity, or to decrease the time required to obtain an assay result.

[0064] The methods of the invention can also be carried out on nucleic acids isolated from specimens and deposited onto solid supports by dot-blotting, or by adsorption onto walls of microtiter plate wells or solid support materials on dipsticks, on membranes, on slides, or on chips as arrays or microarrays.

[0065] Still further, the methods of the invention are applicable to detecting cellular nucleic acids in whole cells from a specimen, such as a fixed or paraffin-embedded section, or from microorganisms immobilized on a solid support, such as replica-plated bacteria or

yeast. In some embodiments, the methods of the invention can be used to make a transcription product corresponding to a target sequence to detect target nucleic acids in living cells.

[0066] A target nucleic acid can be a nucleic acid from any source in purified or unpurified form. For example, a target nucleic acid comprising DNA, can be dsDNA or ssDNA such as mitochondrial DNA, chloroplast DNA, chromosomes, plasmids or other episomes, the genomes of bacteria, yeasts, viruses, viroids, mycoplasma, molds, or other microorganisms, or genomes of fungi, plants, animals, or humans. Target nucleic acids comprising RNA can be tRNA, mRNA, rRNA, mitochondrial RNA, chloroplast RNA, micro RNA, or other RNA molecules, without limit. Target nucleic acids can also be mixtures of DNA and RNA, including, but not limited to, mixtures of the above nucleic acids or fragments thereof, or DNA-RNA hybrids. The target nucleic acid can be only a minor fraction of a complex mixture such as a biological sample and can be obtained from various biological materials by procedures known in the art. Numerous methods for purification of a particular target nucleic are known in the art, if further purification is necessary.

[0067] The term "target nucleic acid sequence" or "target sequence" refers to the particular nucleotide sequence of the target nucleic acid(s) that is/are to be transcribed to make a transcription product. A "target sequence" comprises one or more sequences within one or more target nucleic acids. A target sequence can also have "complexing sequences" which are added during processes of some embodiments of the invention to facilitate joining of a target sequence to another polynucleotide for a particular purpose. For example, a complexing sequence can provide a complementary sequence to which an oligonucleotide (e.g., a primer and/or splice template) used in a method of the invention can anneal or complex. A complexing sequence usually comprises a "tail" sequence that is added by means such as those discussed herein, including, but not limited to, non-templated addition of dCMP residues to first-strand cDNA by reverse transcriptase pausing at cap structures of mRNA (in the presence or absence of manganese cations) and/or controlled ribonucleotide tailing using TdT. If a complexing sequence is added to a target sequence during a process of a method of the invention, it is desirable that a complexing sequence is chosen that does not to affect the specificity of the transcription products made using the resulting transcription substrate comprising the target sequence.

[0068] A target nucleic acid can be either single-stranded or double-stranded RNA, DNA, or mixed RNA and DNA. A target nucleic acid is sometimes referred to more specifically by the type of nucleic acid. By way of example, but not of limitation, a target nucleic acid can be a "target RNA" or an "RNA target," or a "target mRNA," or a "target DNA" or a "DNA target." Similarly, the target sequence can be referred to as "a target RNA sequence" or a "RNA target sequence", or as a "target mRNA sequence" or a "target DNA sequence," or the like. In some embodiments, the target sequence comprises one or more entire target nucleic acids, such as, one or all full-length mRNA molecules in a particular sample. In other embodiments, the target sequence comprises only a portion of one or more target nucleic acid molecules. When the target nucleic acid is originally single-stranded, the term "target sequence" is also meant to refer to the sequence complementary to the "target sequence." When the "target nucleic acid" is originally double-stranded, the term "target sequence" may refer to both the sense strand of the sequence or its complement, or both, depending on the intended purpose of the method. The target sequence may be known or not known, in terms of its actual sequence. In some instances, the terms "target sequence," "target nucleic acid," "target polynucleotide," and variations thereof are used interchangeably.

3. cDNA/First-Strand cDNA/Second-Strand cDNA/Reverse Transcriptase/RNaseH

[0069] In some important embodiments of the invention, the target sequence of a transcription substrate comprises cDNA. In general, "cDNA" refers to "complementary DNA" that is synthesized by primer extension using a DNA polymerase, including, but not limited to, an RNA-dependent DNA polymerase or reverse transcriptase, using at least a portion of a target nucleic acid as a template, and which cDNA is "homologous to" or "base pairs with" at least a portion of the target nucleic acid template. In some embodiments of the invention, which are preferred embodiments, cDNA is obtained by reverse transcription primer extension using a reverse transcriptase and a target nucleic acid comprising messenger RNA (mRNA) obtained from a biological sample as a template, and which cDNA is homologous to the mRNA. Methods in the art related to making cDNA from mRNA involve synthesis of double-stranded cDNA comprising first-strand cDNA and second-strand cDNA, which usually are synthesized sequentially using different methods. However herein, we often refer to "first-strand cDNA" even when a method of the invention results in synthesis of only one strand of DNA that is complementary to the

mRNA (i.e., the term "first-strand cDNA" is not intended to imply that there is also a second-strand cDNA). In some embodiments, the terms "first-strand cDNA" or "cDNA" refer to a single-stranded DNA molecule obtained by reverse transcription of any RNA molecule, even if it is not mRNA. In other embodiments, the terms "first-strand cDNA" or "cDNA" refer to a single-stranded DNA molecule obtained by primer extension using a target nucleic acid comprising either a single-stranded DNA or one strand of a double-stranded DNA as a template for a DNA polymerization reaction.

[0070] If a promoter primer of the invention is used for primer extension of a target sequence, the promoter sequence is at the 5'-end of the resulting first-strand cDNA. Even if the promoter sequence of the promoter primer is a sense promoter sequence, it is not operably joined to the 3'-end of the target sequence as required to obtain a functional double-stranded promoter by complexing with an anti-sense promoter oligo. Therefore, a product obtained by primer extension of a promoter primer is referred to herein simply as "first-strand cDNA" or as a "first-strand cDNA primer extension product" or as a "primer extension product." However, once the promoter sequence is joined to the 3'-end of the target sequence obtained by primer extension, the resulting circular molecule is referred to herein as "circular sense promoter-containing first-strand cDNA." Similarly, the molecule resulting from linearization of circular sense promoter-containing first-strand cDNA to obtain a linear moleculewith a sense promoter on the 3'-end of the target sequence is referred to herein as "linear sense promoter-containing first-strand cDNA." These terms, which designate the presence of a sense promoter at the 3'-end of the target sequence in the respective circular or linear first-strand cDNA, are used so that the reader will understand that a circular or linear transcription substrate, respectively, can be obtained by complexing (or annealing) an anti-sense promoter oligo to the respective circular or linear sense promoter-containing first-strand cDNA.

[0071] Based on a reading of the present description, the reader will understand that a transcription substrate cannot be obtained by annealing a sense promoter oligo to an "anti-sense promoter containing first-strand cDNA" (because the sense promoter sequence must be joined to the 3'-end of a target sequence on the template strand). That is, a functional double-stranded transcription promoter is only obtained by complexing an anti-sense promoter oligo to a sense promoter on the template strand. Therefore, the reader will understand that circularization of anti-sense promoter-containing first-strand cDNA will not yield a transcription substrate by complexing with a sense promoter oligo. However, rolling

circle DNA replication of circular anti-sense promoter-containing first-strand cDNA yields concatemeric "linear sense promoter-containing first-strand cDNA," which can be used to obtain a concatemeric linear transcripton substrate by complexing with an anti-sense promoter oligo. The transcription products are anti-sense transcription products with respect to the target nucleic acid.

[0072] An "RNA-dependent DNA polymerase" or "reverse transcriptase" is an enzyme that can synthesize a complementary DNA copy ("cDNA") from an RNA template. All known reverse transcriptases also have the ability to make a complementary DNA copy from a DNA template; thus, they are both RNA- and DNA-dependent DNA polymerases. A "template" is the nucleic acid molecule that is copied by a nucleic acid polymerase. If the nucleic acid comprises two strands (i.e., is "double-stranded"), and sometimes even if the nucleic acid comprises only one strand (i.e., is "single-stranded"), the strand that is copied is the "template" or "the template strand." The synthesized copy is complementary to the template. Both RNA and DNA are always synthesized in the 5'-to-3' direction and the two strands of a nucleic acid duplex always are aligned so that the 5' ends of the two strands are at opposite ends of the duplex (and, by necessity, so then are the 3' ends). A primer is required for both RNA and DNA templates to initiate synthesis by a DNA polymerase. Examples of reverse transcriptases that can be used in methods of the present invention include, but are not limited to, AMV reverse transcriptase, MMLV reverse transcriptase, Tth DNA polymerase, rBst DNA polymerase large fragment, also called IsoTherm™ DNA Polymerase (Epicentre Technologies, Madison, WI, USA), and BcaBEST™ DNA polymerase (Takara Shuzo Co, Kyoto, Japan). In some cases, a mutant form of a reverse transcriptase, such as, an MMLV reverse transcriptase that lacks RNase H activity is used. In still other embodiments, IsoTherm™ DNA polymerase is most suitable. In other embodiments, a wild-type enzyme is preferred. In general, the invention is not limted with respect to the reverse transcriptase used so long as it functions for its intended purpose.

[0073] In embodiments of the invention that obtain a transcription substrate in which a ssDNA target sequence comprising first-strand cDNA is synthesized using a reverse transcriptase and a primer that is complementary to and anneals to the 3'-end of a target nucleic acid comprising mRNA, the primer can comprise oligo$(dT)_n$ or modified oligo$(dT)_n$, or it can comprise an oligo $d(T)_nX$ anchor primer, wherein "X" comprises either a specific base for a specific mRNA or a randomized nucleotide (i.e., synthesized with a

mixture of all four nucleotides) for priming all mRNA molecules in a sample, or the primer can comprise an oligonucleotide having a specific sequence that is complementary to the sequence of a specific mRNA molecule, or in some cases, it can comprise an oligonucleotide having a random sequence (i.e., synthesized using a mixture of all four nucleotides for every position of the primer).

[0074] If a target nucleic acid is RNA, such as mRNA, and it is desirable to remove the RNA that is annealed to first-strand cDNA following reverse transcription using a sense promoter primer, this can be accomplished by one of several means. By way of example, but not of limitation, the RNA can be removed by treatment with RNase H, by treatment with a base, such as, but not limited to sodium or potassium hydroxide, or the RNA can be removed from the hybrid by heat denaturation. In preferred embodiments for some applications, the RNA is removed by an RNase H activity of a reverse transcriptase that is used for reverse transcription (or primer extension), such as, but not limited to, MMLV reverse transcriptase. Alternatively, in some embodiments, the RNA is dissociated from the first-strand cDNA by incubating the hybrid or performing the reverse transcription in the presence of a single-strand binding (SSB) protein of the invention, such as, but not limited to *E. coli* SSB (*Eco*SSB).

[0075] In some embodiments of the invention, especially in embodiments for obtaining additional rounds of transcription products, a separate RNase H enzyme is also used, whether or not the reverse transcriptase has RNase H activity. If RNase H activity is desirable in an embodiment for obtaining multiple rounds of transcription, but a separate RNase H enzyme is not added, MMLV reverse transcriptase (wild-type RNase H-positive) can be used. AMV reverse transcriptase can be used in some embodiments for obtaining multiple rounds of transcription in which a separate RNase H enzyme is also added. Kacian et al. (U.S. Patent No. 5,399,491) disclose information related to the effects of adding different amounts of a separate RNase H enzyme to transcription-mediated amplification assays that use either MMLV or AMV reverse transcriptase, which reference and information is incorporated herein by reference and made a part of the present disclosure.

[0076] "Ribonuclease H" or "RNase H" is an enzyme that degrades the RNA portion of an RNA:DNA duplex. An RNase H can be an endonuclease or an exonuclease. Most wild-type reverse transcriptase enzymes have an RNase H activity in addition to their polymerase activity. However, other sources of the RNase H are available without an associated polymerase activity. The degradation may result in separation of RNA from an

RNA:DNA complex. Alternatively, the RNase H may simply cut the RNA at various locations such that portions of the RNA melt off or permit enzymes to unwind portions of the RNA. When used in an embodiment of the invention, RNaseH enzymes that can be used include, but are not limited to, *E. coli* RNase H, *Thermus thermophilus* RNase H, and *Thermus flavus* RNase H (U.S. Patents Nos. 5,268,289; 5,459,055; and 5,500,370, incorporated herein by reference). The latter two enzymes, which are thermostable and, therefore, maintain more consistent activity in reactions and are more easily stored and shipped, are preferred in most embodiments in which a separate RNase H enzyme is used. Other RNase H enzymes that can be used are those that are described by Sagawa et al. in PCT Patent Publication No. WO 02/16639; and in PCT Patent Publications Nos. WO 00/56877 and AU 00/29742, all of which are incorporated herein by reference. In other embodiments, it is desirable to use a less thermally stable enzyme, such as *E. coli* RNase H, because it is easier to inactivate the enzyme in a reaction mixture.

[0077] Kacian et al. disclosed in U.S Patent No. 5,399,491, incorporated herein by reference, that the number, distribution, and position of putative RNase H cut sites determine, in part, the usefulness of a given primer and that amplification can be improved by inclusion of intentional mismatches or insertion of sequences in order to affect the number, distribution, and position of putative RNase H cut sites. Thus, in preferred processes of the invention for removing RNA from RNA:DNA hybrids following reverse transcription to make first-strand cDNA, the RNA target sequence is determined and then analyzed to determine where RNase H degradation will cause cuts or removal of sections of RNA from the duplex upon synthesis of first-strand cDNA. The processes of the invention include conducting experiments to determine the effect on amplification of the target sequence of the degradation of the RNA target sequence by RNase H present in the reverse transcriptase and/or separate RNase H enzyme(s) used, including, but not limited to, AMV reverse transcriptase, and both RNase H-plus and RNase H-minus MMLV reverse transcriptase, and *E. coli* RNase H or thermostable RNase H enzymes that are stable for more than 10 minutes at 70° C (U.S. Patents Nos. 5,268,289; 5,459,055; and 5,500,370, incorporated herein by reference), such as, but not limited to, Hybridase™ thermostable RNase H (Epicentre Technologies, Madison, WI, USA), Tth RNase H, and Tfl RNase H, or by different combinations of a reverse transcriptase and a separate RNase H.

[0078] In selecting a primer, including a promoter primer of the invention, for use in reverse transcription of an RNA target sequence to make first-strand cDNA, it is preferable

that the primer be selected so that it will hybridize to a section of RNA which is substantially nondegraded by the RNase H present in the reaction mixture. If there is substantial degradation, the cuts in the RNA strand in the region of the primer may stop or inhibit DNA synthesis and prevent extension of the primer. Thus, it is desirable to select a primer that will hybridize with a sequence of the RNA target, located so that when the RNA is subjected to RNase H, there is no substantial degradation that would prevent formation of the primer extension product.

4. Transcription Substrate

[0079] As used herein, a "transcription substrate" according to the present invention comprises a target sequence that is operably joined to a transcription promoter, wherein an RNA polymerase can bind to the transcription promoter with specificity and synthesize a transcription product corresponding to the target sequence under suitable transcription conditions. In order to be operably joined to the target sequence, a sense transcription promoter is 3'-of the target sequence and, if the respective promoter for the RNA polymerase must be double-stranded to be functional, then an oligo comprising an anti-sense promoter sequence is annealed to the sense promoter sequence that is joined to the target sequence. If the sense transcription promoter comprises a single-stranded pseudopromoter or synthetic promoter that has been selected or identified, as described elsewhere herein, then the transcription substrate can comprise single-stranded DNA (i.e., without annealing an anti-sense promoter oligo).

[0080] A transcription substrate of the invention can also have additional nucleic acid sequences that are 5'-of and/or 3'-of the transcription promoter sequence, but a transcription substrate is not required to have such additional other sequences. By way of example, but not of limitation, a transcription substrate can have a transcription initiation site 5'-of the promoter sequence. Also, in some embodiments of the invention, a transcription substrate can have one or more transcription termination sequences, one or more sites for DNA cleavage to permit controlled linearization of a circular first-strand cDNA that can be used to obtain a linear transcription substrate as described elsewhere herein, one or more origins ("*ori*'s") of replication (preferably an *ori* for a single-stranded replicon, such as, but not limited to, a phage M13 replicon), a selectable or screenable marker, such as, but not limited to an antibiotic-resistance gene or a beta-galactosidase gene, respectively, or one or more transposon recognition sequences, such as outer end

("OE") or mosaic end ("ME") sequences for a Tn5-type transposon, that, in double-stranded form, can be recognized and used by a transposase for *in vitro* or *in vivo* transposition, or one or more sites that are recognized by a recombinase (such as, but not limited to, the cre-lox system), and/or other sequences and genetic elements for a particular purpose, including, but not limited to, sequences that are transcribed by the RNA polymerase so as to provide additional regions of complementarity in the RNA transcription products for annealing of primers for reverse transcription in order to make cDNA for additional rounds of transcription. In most embodiments, the target sequence or template portion of a transcription substrate of the present invention is single-stranded, whereas double-stranded DNA templates are used in other methods in the art for obtaining transcription products corresponding to a target sequence.

[0081] Since, except for the promoter sequence, a transcription substrate of the present invention is single-stranded, the terms "3'-of" and "5'-of" are used herein with respect to the present invention to refer to the position or orientation of a particular nucleic acid sequence or genetic element, such as, but not limited to, a transcription promoter, relative to other sequences or genetic elements within the DNA template strand comprising the transcription substrate. Thus, although the synthesis of RNA in a 5'-to-3' direction during transcription is thought of as proceeding in a "downstream" direction, the sense transcription promoter sequence on the transcription substrate is referred to herein as being 3'-of the target sequence. Those with knowledge in the art will understand these terms in the context of nucleic acid chemistry and structure, particularly related to the 3'- and 5'-positions of sugar moieties of canonical nucleic acid nucleotides. By way of example, a sense transcription promoter that is "3'-of the target sequence" on a linear transcription substrate refers to a sense promoter sequence that is at or closer to the 3'-end of the transcription substrate relative to the target sequence on the same strand. If a first nucleic acid sequence is 3'-of a second sequence on one strand, the complement of the first sequence will be 5'-of the complement of the second sequence on the complementary strand. The description of the invention will be understood with respect to the relative 5' or 3' position and orientation of a sequence or genetic element within a particular nucleic acid strand, unless explicitly stated to the contrary.

5. Nucleic Acids, Polynucleotides and Analogs Thereof

[0082] A "nucleic acid" or "polynucleotide" of the invention is a polymer molecule comprising a series of "mononucleosides," also referred to as "nucleosides," in which the 3'-position of the pentose sugar of one nucleoside is linked by an internucleoside linkage, such as, but not limited to, a phosphodiester bond, to the 5'-position of the pentose sugar of the next nucleoside. A nucleoside linked to a phosphate group is referred to as a "nucleotide." The nucleotide that is linked to the 5'-position of the next nucleotide in the series is referred to as "5' of" or the "5' nucleotide" and the nucleotide that is linked to the 3'-position of the 5' nucleotide is referred to as "3' of" or the "3' nucleotide." The pentose sugar of the nucleic acid can be ribose, in which case, the nucleic acid or polynucleotide is referred to as "RNA," or it can be 2'-deoxyribose, in which case, the nucleic acid or polynucleotide is referred to as "DNA." Alternatively, especially if the nucleic acid is synthesized chemically, the nucleic acid can be composed of both DNA and RNA mononucleotides. In both RNA and DNA, each pentose sugar is covalently linked to one of four common or "canonical" nucleic acid bases (each also referred to as a "base"). Three of the predominant naturally-occurring bases that are linked to the sugars (adenine, cytidine and guanine) are common for both DNA and RNA, while one base is different; DNA has the additional base thymine, while RNA has the additional base uridine. Those in the art commonly think of a small polynucleotide as an "oligonucleotide." The term "oligonucleotide" as used herein is defined as a molecule comprised of two or more deoxyribonucleotides or ribonucleotides, preferably about 10 to 200 nucleotides, but there is no defined limit to the length of an oligonucleotide. The exact size will depend on many factors, which in turn depends on the ultimate function or use of the oligonucleotide.

[0083] In order to accomplish the goals of the invention, there is no limit to the composition of the nucleic acids or polynucleotides of the invention including any splice template oligos, primers, including promoter primers, ligation splint oligos, detection probes, such as, but not limited to molecular beacons (U.S. Patents Nos. 5,925,517 and 6,103,476 of Tyagi et al. and 6,461,817 of Alland et al., which are incorporated herein by reference), capture probes, oligonucleotides, or other nucleic acids used or detected in the assays or methods, so long as each nucleic acid functions for its intended use. By way of example, but not of limitation, the nucleic acid bases in the mononucleotides may comprise guanine, adenine, uracil, thymine, or cytidine, or alternatively, one or more of the nucleic acid bases may comprise xanthine, allyamino-uracil, hypoxanthine, 2-aminoadenine, 6-

methyl and other alkyl adenines, 2-propyl and other alkyl adenines, 5-halouracil, 5-halo cytosine, 5-propynyl uracil, 5-propynyl cytosine, 7-deazaadenine, 7-deazaguanine, 7-deaza-7-methyl-adenine, 7-deaza-7-methyl-guanine, 7-deaza-7-propynyl-adenine, 7-deaza-7-propynyl-guanine and other 7-deaza-7-alkyl or 7-aryl purines, N2-alkyl-guanine, N2-alkyl-2-amino-adenine, purine 6-aza uracil, 6-aza cytosine and 6-aza thymine, 5-uracil (pseudo uracil), 4-thiouracil, 8-halo adenine, 8-amino-adenine, 8-thiol adenine, 8-thiolalkyl adenines, 8-hydroxyl adenine and other 8-substituted adenines and 8-halo guanines, 8-amino-guanine, 8-thiol guanine, 8-thiolalkyl guanines, 8-hydroxyl guanine and other 8-substituted guanines, other aza and deaza uracils, other aza and deaza thymidines, other aza and deaza cytosine, aza and deaza adenines, aza and deaza guanines or 5-trifluoromethyl uracil and 5-trifluorocytosine. Still further, they may comprise a nucleic acid base that is derivatized with a biotin moiety, a digoxigenin moiety, a fluorescent or chemiluminescent moiety, a quenching moiety or some other moiety. The invention is not limited to the nucleic acid bases listed; this list is given to show the broad range of bases which may be used for a particular purpose in a method.

[0084] In some embodiments of the invention, a molecule comprising a "peptide nucleic acid" (PNA) or a molecule comprising both a nucleic acid and a PNA, as described in U.S Pat. Nos. 5,539,082; 5,641,625; 5,700,922; 5,705,333; 5,714,331; 5,719,262; 5,736,336; 5,773,571; 5,786,461; 5,817,811; 5,977,296; 5,986,053; 6,015,887; and 6,020,126 (and references therein), can also be used. In general, a PNA molecule is a nucleic acid analog consisting of a backbone comprising, for example, N-(2-aminoethyl)glycine units, to each of which a nucleic acid base is linked through a suitable linker, such as, but not limited to an aza, amido, ureido, or methylene carbonyl linker. The nucleic acid bases in PNA molecules bind complementary single-stranded DNA or RNA according to Watson-Crick base-pairing rules. However, the T_m 's for PNA/DNA or PNA/RNA duplexes or hybrids are higher than the T_m's for DNA/DNA, DNA/RNA, or RNA/RNA duplexes. PNA provides tighter binding and greater binding stability than a nucleic acid of similar base sequence (e.g., see U.S. Pat. No. 5,985,563). Also, since PNA is not naturally occurring, PNA molecules are highly resistant to protease and nuclease activity. PNA can be prepared according to methods know in the art, such as, but not limited to, methods described in the above-mentioned patents, and references therein.

[0085] When a molecule comprising both a nucleic acid and a peptide nucleic acid (PNA) is used in the invention, modified bases can be used in one or both parts. For

example, binding affinity can be increased by the use of certain modified bases in both the nucleotide subunits that make up the 2'-deoxyoligonucleotides of the invention and in the peptide nucleic acid subunits. Such modified bases may include 5-propynylpyrimidines, 6-azapyrimidines, and N-2, N-6 and O-6 substituted purines including 2-aminopropyl-adenine. Other modified pyrimidine and purine base are also expected to increase the binding affinity of macromolecules to a complementary strand of nucleic acid.

[0086] With respect to nucleic acids or polynucleotides of the invention, one or more of the sugar moieties can comprise ribose or 2'-deoxyribose, or alternatively, one or more of the sugar moieties can be some other sugar moiety, such as, but not limited to, 2'-fluoro-2'-deoxyribose or 2'-O-methyl-ribose, which provide resistance to some nucleases, or 2'-amino-2'-deoxyribose or 2'-azido-2'-deoxyribose, which can be used to label transcription products by reacting them with visible, fluorescent, infrared fluorescent or other detectable dyes or chemicals having an electrophilic, photoreactive or other reactive chemical moiety.

[0087] The internucleoside linkages of nucleic acids or polynucleotides of the invention can be phosphodiester linkages, or alternatively, one or more of the internucleoside linkages can comprise modified linkages, such as, but not limited to, phosphorothioate, phosphorodithioate, phosphoroselenate, or phosphorodiselenate linkages, which are resistant to some nucleases.

[0088] A variety of methods are known in the art for making nucleic acids having a particular sequence or that contain particular nucleic acid bases, sugars, internucleoside linkages, chemical moieties, and other compositions and characteristics. Any one or any combination of these methods can be used to make a nucleic acid, polynucleotide, or oligonucleotide for the present invention. The methods include, but are not limited to:

[0089] (1) chemical synthesis (usually, but not always, using a nucleic acid synthesizer instrument);

[0090] (2) post-synthesis chemical modification or derivatization;

[0091] (3) cloning of a naturally occurring or synthetic nucleic acid in a nucleic acid cloning vector (e.g., see Sambrook, et al., Molecular Cloning: A Laboratory Approach Second Edition, 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY and Sambrook and Russell, Molecular Cloning, A Laboratory Manual, Third Edition, 2001, Cold Spring Harbor Laboratory Press,) such as, but not limited to a plasmid, bacteriophage (e.g., M13 or lamba), phagemid, cosmid, fosmid, YAC, or BAC cloning vector, including vectors for producing single-stranded DNA;

[0092] (4) primer extension using an enzyme with DNA template-dependent DNA polymerase activity, such as, but not limited to, Klenow, T4, T7, r*Bst*, *Taq*, *Tfl*, or *Tth* DNA polymerases, including mutated, truncated (e.g., exo-minus), or chemically-modified forms of such enzymes;

[0093] (5) PCR (e.g., see Dieffenbach, C.W., and Dveksler, eds., PCR Primer: A Laboratory Manual, 1995, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY);

[0094] (6) reverse transcription (including both isothermal synthesis and RT-PCR) using an enzyme with reverse transcriptase activity, such as, but not limited to, reverse transcriptases derived from avian myeloblasosis virus (AMV), Maloney murine leukemia virus (MMLV), *Bacillus stearothermophilus* (r*Bst*), or T*hermus thermophilus* (*Tth*);

[0095] (7) *in vitro* transcription using an enzyme with RNA polymerase activity, such as, but not limited to, SP6, T3, or T7 RNA polymerase, *Tth* RNA polymerase, *E. coli* RNA polymerase, or SP6 or T7 R&DNA™ Polymerase (Epicentre Technologies, Madison, WI, USA), or another enzyme;

[0096] (8) use of restriction enzymes and/or modifying enzymes, including, but not limited to exo- or endonucleases, kinases, ligases, phosphatases, methylases, glycosylases, terminal transferases, including kits containing such modifying enzymes and other reagents for making particular modifications in nucleic acids;

[0097] (9) use of polynucleotide phosphorylases to make new randomized nucleic acids;

[0098] (10) other compositions, such as, but not limited to, a ribozyme ligase to join RNA molecules; and/or

[0099] (11) any combination of any of the above or other techniques known in the art.

[00100] Oligonucleotides and polynucleotides, including chimeric (i.e., composite) molecules and oligonucleotides with modified bases, sugars, or internucleoside linkages are commercially available (e.g., TriLink Biotechnologies, San Diego, CA, USA or Integrated DNA Technologies, Coralville, IA).

[00101] A "portion" or "region," used interchangeably herein, of a polynucleotide or oligonucleotide is a contiguous sequence of 2 or more bases. In other embodiments, a region or portion is at least about any of 3, 5, 10, 15, 20, 25 contiguous nucleotides.

6. Ligation / Ligase

[00102] "Ligation" refers to the joining of a 5'-phosphorylated end of one nucleic acid molecule to a 3'-hydroxyl end of the same or another nucleic acid molecule by an

enzyme called a "ligase." Alternatively, in some embodiments of the invention, ligation is effected by a type I topoisomerase moiety attached to one end of a nucleic acid (see U.S. Patent No. 5,766,891, incorporated herein by reference). The terms "ligating," "ligation," and "ligase" are often used in a general sense herein and are meant to comprise any suitable method and composition for joining a 5'-end of one nucleic acid to a 3'-end of the same or another nucleic acid. Different ligases are preferred in different embodiments of the invention, as discussed elsewhere herein.

[00103] In general, if a nucleic acid to be ligated comprises RNA, a ligase such as, but not limited to, T4 RNA ligase, a ribozyme ligase, Tsc RNA Ligase (Prokaria Ltd., Reykjavik, Iceland), or another ligase can be used for non-homologous joining of the ends. T4 DNA ligase can also be used to ligate RNA molecules when a 5'-phosphoryl end is adjacent to a 3'-hydroxyl end annealed to a complementary sequence (e.g., see U.S. Patent No. 5,807,674 of Tyagi, incorporated herein by reference).

[00104] If the nucleic acids to be joined comprise DNA and the 5'-phosphorylated and the 3'-hydroxyl ends are ligated when the ends are annealed to a complementary DNA so that the ends are adjacent (such as, when a "ligation splint" or "ligation splint oligo" is used), then enzymes such as, but not limited to, T4 DNA ligase, Ampligase® DNA Ligase (Epicentre Technologies, Madison, WI, USA), Tth DNA ligase, Tfl DNA ligase, or Tsc DNA Ligase (Prokaria Ltd., Reykjavik, Iceland) can be used. However, the invention is not limited to the use of a particular ligase and any suitable ligase can be used. Still further, Faruqui discloses in U.S. Patent No. 6,368,801 that T4 RNA ligase can efficiently ligate DNA ends of nucleic acids that are adjacent to each other when hybridized to an RNA strand. Thus, T4 RNA ligase is a suitable ligase of the invention in embodiments in which DNA ends are ligated on a ligation splint oligo comprising RNA or modified RNA, such as, but not limited to modified RNA that contains 2'-F-dCTP and 2'-F-dUTP made using the DuraScribe™ T7 Transcription Kit (Epicentre Technologies, Madison, WI, USA). With respect to ligation on an homologous ligation template, especially ligation using a "ligation splint" or a "ligation splint oligo" (as discussed elsewhere herein), a region, portion, or sequence that is "adjacent" to another sequence directly abuts that region, portion, or sequence.

[00105] In other embodiments comprising intramolecular ligation of linear ssDNA, ligation can be effected in the absence of a ligation splint using a ligase that can catalyze non-homologous ligation of ssDNA, such as, but not limited to, ThermoPhage™

RNA Ligase II (Prokaria Ltd., Reykjavik, Iceland), which is derived from phage TS2126 that infects Thermus scotoductus.

7. DNA Polymerases/Strand-Displacing DNA Polymerases/Strand Displacement/Rolling Circle Replication

[00106] A "DNA-dependent DNA polymerase" is an enzyme that synthesizes a complementary DNA ("cDNA") copy from a DNA template. Examples are DNA polymerase I from *E. coli* and bacteriophage T7 DNA polymerase. All known DNA-dependent DNA polymerases require a complementary primer to initiate synthesis. It is known that under suitable conditions a DNA-dependent DNA polymerase may synthesize (i.e., "reverse transcribe") a complementary DNA copy from an RNA template, a process that is also referred to as "reverse transcription," for which application the DNA polymerase can also be referred to as a "reverse transcriptase."

[00107] Some DNA polymerases are able to displace the strand complementary to the template strand as a new DNA strand is synthesized by the polymerase. This process is called "strand displacement" and the DNA polymerases that have this activity are referred to herein as "strand-displacing DNA polymerases." The template for strand displacement DNA synthesis using a method of the invention can be a linear or circular ssDNA. If the DNA template is a single-stranded circle, primed DNA synthesis procedes around and around the circle, with continual displacement of the strand ahead of the replicating strand, a process called "rolling circle replication." Rolling circle replication results in synthesis of tandem copies of the circular template. The suitability of a DNA polymerase for use in an embodiment of the invention that comprises strand displacement on linear templates or rolling circle replication can be readily determined by assessing its ability to carry out rolling circle replication. By way of example, but not of limitation, the ability of a polymerase to carry out rolling circle replication can be determined by using the polymerase in a rolling circle replication assay such as those described by Fire and Xu (Proc. Natl. Acad. Sci. USA 92:4641-4645, 1995), incorporated herein by reference. It is preferred that a DNA polymerase be a strand displacing DNA polymerase and lack a 5'-to-3' exonuclease activity for strand displacement polymerization reactions using both linear or circular templates since a 5'-to-3' exonuclease activity, if present, might result in the destruction of the synthesized strand. It is also preferred that DNA polymerases for use in the disclosed strand displacement synthesis methods are highly processive. The ability of a DNA polymerase to strand-displace can vary with reaction conditions, in addition to the

particular enzyme used. Strand displacement and DNA polymerase processivity can also be assayed using methods described in Kong et al. (J. Biol. Chem. 268:1965-1975, 1993 and references cited therein, all of which are incorporated herein by reference).

[00108] Preferred strand displacing DNA polymerases of the invention are rBst DNA polymerase large fragment (also called IsoTherm™ DNA Polymerase (EPICENTRE Technologies, Madison, WI, USA), BcaBEST™ DNA polymerase (Takara Shuzo Co., Kyoto, Japan), RepliPHI™ DNA Polymerase (EPICENTRE Technologies, Madison, WI, USA), ϕ29 DNA polymerase (U.S. Pat. Nos. 5,576,204 and 5,001,050), SequiTherm™ DNA polymerase (Epicentre Technologies, Madison, WI, USA), and MMLV reverse transcriptase. Other strand-displacing DNA polymerases which can be used include, but are not limited to, phage M2 DNA polymerase (Matsumoto et al., Gene 84:247, 1989), phage ϕ PRD1 DNA polymerase (Jung et al., Proc. Natl. Acad. Sci. USA 84: 8287, 1987), VENT® DNA polymerase (Kong et al., J. Biol. Chem. 268:1965-1975, 1993), Klenow fragment of DNA polymerase I (Jacobsen et al., Eur. J. Biochem. 45:623-627, 1974), T5 DNA polymerase (Chatterjee et al., Gene 97:13-19, 1991), PRD1 DNA polymerase (Zhu and Ito, Biochim. Biophys. Acta 1219: 267-276, 1994), modified T7 DNA polymerase (Tabor and Richardson, J. Biol. Chem. 262:15,330-15,333, 1987); Tabor and Richardson, J. Biol. Chem. 264:6447-6458, 1989); Sequenase™ (U.S. Biochemicals, Cleveland, OH, USA), and T4 DNA polymerase holoenzyme (Kaboord and Benkovic, Curr. Biol. 5:149-157, 1995), all of which references, are incorporated herein by reference. Strand displacing DNA polymerases are useful in some embodiments of the invention for strand-displacing rolling circle replication of circular first-strand cDNA or circular second-strand cDNA. IsoTherm™ DNA polymerase (rBst DNA polymerase large fragment; Epicentre) is most preferred because, in addition to having strand-displacing DNA polymerase activity, it can also be used as a reverse transcriptase for synthesis of first-strand cDNA from RNA target nucleic acids (e.g., U.S. Patent No. 6,030,814 of Jendrisak et al). BcaBEST™ DNA polymerase (Takara Shuzo Co., Kyoto, Japan) can also be used as a reverse transcriptase as well as a strand-displacing DNA polymerase.

[00109] Strand displacement can be facilitated through the use of a strand displacement factor, such as helicase. It is considered that any DNA polymerase that can perform rolling circle replication in the presence of a strand displacement factor is suitable for use in embodiments of the invention that comprise strand displacement or rolling circle replication, even if the DNA polymerase does not perform rolling circle replication in the

absence of such a factor. Strand displacement factors useful in rolling circle replication include, but are not limited to, BMRF1 polymerase accessory subunit (Tsurumi et al., J. Virology 67:7648-7653, 1993), adenovirus DNA-binding protein (Zijderveld and van der Vliet, J. Virology 68:1158-1164, 1994), herpes simplex viral protein ICP8 (Boehmer and Lehman, J. Virology 67:711-715, 1993); Skaliter and Lehman, Proc. Natl. Acad. Sci. USA 91:10,665-10,669, 1994), single-stranded DNA binding proteins (SSB; Rigler and Romano, J. Biol. Chem. 270:8910-8919, 1995), and calf thymus helicase (Siegel et al., J. Biol. Chem. 267:13,629-13,635, 1992).

8. Hybridize/Hybridization

[00110] The terms "hybridize" and "hybridization" refer to the formation of complexes between nucleotide sequences that are sufficiently complementary to form complexes via Watson-Crick base pairing. With respect to the present invention, nucleic acid sequences that "hybridize" or "anneal" with each other should form "hybrids" or "complexes" that are sufficiently stable to serve the intended purpose. By way of example, but not of limitation, where a primer or splice template oligo hybridizes or anneals with a target nucleic acid in a sample or with a "tailed" target sequence, respectively, each respective complex or hybrid should be sufficiently stable to serve the respective priming functions required for a DNA polymerase to copy the target sequence by primer extension of the annealed primer or to extend the 3'-end of the target sequence using the annealed splice template oligo as a template, respectively.

9. RNA Polymerase Promoters

[00111] In certain embodiments of the invention, promoter sequences may be used that that are recognized specifically by a DNA-dependent RNA polymerase, such as, but not limited to, those described by Chamberlin and Ryan, In: The Enzymes. San Diego, Calif., Academic Press, 15:87-108, 1982, and by Jorgensen et al., J. Biol. Chem. 266:645-655, 1991. Several RNA polymerase promoter sequences are especially useful, including, but not limited to, promoters derived from SP6 (e.g., Zhou and Doetsch, Proc. Nat. Acad. Sci. USA 90:6601-6605, 1993), T7 (e.g., Martin, and Coleman, Biochemistry 26:2690-2696, 1987) and T3 (e.g., McGraw et al., Nucl. Acid. Res. 13:6753-6766, 1985). An RNA polymerase promoter sequence derived from *Thermus thermophilus* can also be used (see,

e.g., Wendt et al., Eur. J. Biochem. 191:467-472, 1990; Faraldo et al., J. Bact. 174:7458-7462, 1992; Hartmann et al., Biochem. 69:1097-1104, 1987; Hartmann et al., Nucl. Acids Res. 19:5957-5964, 1991). The length of the promoter sequence will vary depending upon the promoter chosen. For example, the T7 RNA polymerase promoter can be only about 25 bases in length and act as a functional promoter, while other promoter sequences require 50 or more bases to provide a functional promoter.

[00112] In other embodiments of the invention, a promoter is used that is recognized by an RNA polymerase from a T7-like bacteriophage. The genetic organization of all T7-like phages that have been examined has been found to be essentially the same as that of T7. Examples of T7-like phages according to the invention include, but are not limited to *Escherichia coli* phages T3, φI, φII, W31, H, Y, A1, 122, cro, C21, C22, and C23; *Pseudomonas putida* phage gh-1; *Salmonella typhimurium* phage SP6; *Serratia marcescens* phages IV; Citrobacter phage ViIII; and *Klebsiella* phage No. 11 (Hausmann, Current Topics in Microbiology and Immunology 75:77-109, 1976; Korsten et al., J. Gen. Virol. 43:57-73, 1975; Dunn, et al., Nature New Biology 230:94-96, 1971; Towle, et al., J. Biol. Chem. 250:1723-1733, 1975; Butler and Chamberlin, J. Biol. Chem. 257:5772-5778, 1982).

[00113] Some embodiments of the invention also comprises use of the coliphage N4 RNA polymerase (N4 vRNAP) (Rothman-Denes, L.B., and Schito, G.C., Virology, 60: 65-72, 1974; Falco, S.C. *et al.*, Proc. Natl. Acad. Sci. USA, 74: 520-523, 1977; Falco, S.C. *et al.*, J. Biol. Chem., 255: 4339-4347, 1980; Kazmierczak, K.M., et al., EMBO J., 21: 5815-5823, 2002, all of which are incorporated herein by reference) and the promoter sequences of said ssDNA oligos comprise a conserved promoter sequence recognized by the *Escherichia coli* phage N4 vRNAP, wherein said promoter sequence comprises a 5-basepair stem and 3-base loop hairpin structure (Glucksmann, M.A. *et al.*, Cell, 70: 491-500, 1992; Haynes,L.L. and Rothman-Denes, L.B., Cell, 41: 597-605, 1985), which references and which promoters are incorporated herein as part of the invention by reference. By way of example, but not of limitation, promoters comprising the following sequences can be used:

[00114] P1: 3'-CAACGAAGCGTTGAATACC T-5'; or

[00115] P2: 3'-TTCTTCGAGGCGAAGAAAACCT -5'; or

[00116] P3: 3'-CGACGAGGCGTCGAAAACCA-5'.

[00117] In contrast to other known RNA polymerases, the N4 vRNAP transcribes single-stranded, promoter-containing templates *in vitro* with *in vivo* specificity (Falco,S.C *et al.*, Proc. Natl. Acad. Sci. USA, 75: 3220-3224, 1978; Haynes, L.L. and Rothman-Denes, L.B., Cell, 41: 597-605, 1985, all of which are incorporated herein by reference). In most preferred embodiments of the invention, the RNA polymerase comprises a transcriptionally active 1,106-amino acid domain of the N4 vRNAP (herein designated "mini-vRNAP"), which corresponds to amino acids 998-2103 of N4 vRNAP (Krystyna M. Kazmierczak, Ph.D. Dissertation, University of Chicago, 2001; Kazmierczak, K.M., et al., EMBO J., 21: 5815-5823, 2002, incorporated herein by reference), and reaction conditions are as described therein.

10. Reaction Conditions

[00118] Appropriate reaction media and conditions for carrying out the methods of the present invention are those that permit nucleic acid transcription and other reactions according to the methods of the present invention. With respect to transcription reactions of the invention with a wild-type or mutant T7 RNAP enzymes, the reaction conditions for *in vitro* transcription are those provided with the AmpliScribe™ T7-Flash™ Transcription Kit, or the AmpliScribe™ T7 High Yield Transcription Kit, or the DuraScribe™ T7 Transcription Kit or, for incorporation of 2'-substituted deoxyribonucleotides other than 2'-fluorine-substituted deoxyribonucleotides, with the T7 R&DNA™ Polymerase, in each case according to the instructions of the manufacturer EPICENTRE Technologies, Madison, WI). If a T3 or SP6 RNAP is used for transcription using a method of the invention, the reaction conditions for *in vitro* transcription are those provided with the AmpliScribe™ T3 High Yield Transcription Kit or with the AmpliScribe™ T3-Flash™ High Yield Transcription Kit, or with the AmpliScribe™ SP6 High Yield Transcription Kit, in each case according to the instructions of the manufacturer EPICENTRE Technologies, Madison, WI).

[00119] If mini-vRNAP or mini-vRNAP Y678F enzymes are used *in vitro* transcription of a single-stranded DNA template having a single-stranded promoter, such as, but not limited to uses for obtaining additional amplification of transcription products resulting from an RCT Signal Probe, the following *in vitro* transcription reaction is prepared by setting up a reaction mixture containing the following final concentrations of components, added in the order given: 0.1 micromolar of a N4 vRNAP promoter-

containing DNA oligo; 1.0 micromolar *Eco*SSB Protein; 1X transcription buffer comprising 40 mM Tris–HCl (pH 7.5), 6 mM $MgCl_2$., 2 mM spermidine, and 10 mM NaCl; 1 mM DTT; 0.5 mM of each NTP (ATP, CTP, GTP and UTP); deionized RNase-free water so the final volume will be 50 microliters after addition of an RNAP; and 0.1 micromolar of mini-vRNAP or mini-vRNAP Y678F enzyme. In some embodiments of the invention, 2'-F-dUTP and 2'-F-dCTP are used at a final concentration of 0.5 mM each in place of UTP and CTP in order to obtain synthesis of modified RNA which is resistant to ribonuclease A-type enzymes. Other modified nucleoside triphosphates can also be used in place of or in addition to the canonical NTPs for specific applications. The reaction mixture is then incubated at 37° C to permit synthesis of RNA from the template. The reaction can be followed by gel electrophoresis on a PAGE gel.

[00120] Reaction conditions for *in vitro* transcription using other RNA polymerases are well known in the art and can be obtained from the public literature.

[00121] The invention is not limited to these reaction conditions or concentrations of reactants. Those with skill in the art will know that other suitable reaction conditions under which an RNA polymerase of the invention can be used can be found by simple experimentation, and any of these reaction conditions are also included within the scope of the invention. Such media and conditions are known to persons of skill in the art, and are described in various publications, such as U.S. Pat. No. 5,679,512 and PCT Pub. No. WO99/42618, incorporated herein by reference. For example, a buffer can be Tris buffer, although other buffers can also be used as long as the buffer components are non-inhibitory to enzyme components of the methods of the invention. The pH is preferably from about 5 to about 11, more preferably from about 6 to about 10, even more preferably from about 7 to about 9, and most preferably from about 7.5 to about 8.5. The reaction medium can also include bivalent metal ions such as Mg^{+2} or Mn^{+2}, at a final concentration of free ions that is within the range of from about 0.01 to about 10 mM, and most preferably from about 1 to 6 mM. The reaction medium can also include other salts, such as KCl, that contribute to the total ionic strength of the medium. For example, the range of a salt such as KCl is preferably from about 0 to about 100 mM, more preferably from about 0 to about 75 mM, and most preferably from about 0 to about 50 mM. The reaction medium can further include additives that could affect performance of the reactions, but that are not integral to the activity of the enzyme components of the methods. Such additives include proteins such as BSA, and non-ionic detergents such as NP40 or Triton. Reagents, such as DTT,

that are capable of maintaining activities enzyme with sulfhydryl groups can also be included. Such reagents are known in the art. Where appropriate, an RNase inhibitor, such as, but not limited to a placental ribonuclease inhibitor (e.g., RNasin®, Promega Corporation, Madison, WI, USA) or an antibody RNase inhibitor, that does not inhibit the activity of an RNase employed in the method can also be included. Any aspect of the methods of the present invention can occur at the same or varying temperatures. Preferably, the reactions are performed isothermally, which avoids the cumbersome thermocycling process. The reactions are carried out at a temperature that permits hybridization of the oligonucleotides of the present invention to the target sequence and/or first-strand cDNA of a method of the invention and that does not substantially inhibit the activity of the enzymes employed. The temperature can be in the range of preferably about 25° C to about 85° C, more preferably about 30° C to about 75° C, and most preferably about 37° C to about 70° C. In the processes that include RNA transcription, the temperature for the transcription steps is lower than the temperature(s) for the preceding steps. In these processes, the temperature of the transcription steps can be in the range of preferably about 25° C to about 85° C, more preferably about 30° C to about 75° C, and most preferably about 37° C to about 55° C.

[00122] As disclosed in U.S. Patent Nos. 6,048,696 and 6,030,814, as well as in German Patent No. DE4411588C1, all of which are incorporated herein by reference and made part of the present invention, it is preferred in many embodiments to use a final concentration of about 0.25 M, about 0.5 M, about 1.0 M, about 1.5 M, about 2.0 M, about 2.5 M or between about 0.25 M and 2.5 M betaine (trimethylglycine) in DNA polymerase or reverse transcriptase reactions in order to decrease DNA polymerase stops and increase the specificity of reactions which use a DNA polymerase.

[00123] Nucleotide and/or nucleotide analogs, such as deoxyribonucleoside triphosphates, that can be employed for synthesis of reverse transcription or primer extension products in the methods of the invention are provided in an amount that is determined to be optimal or useful for a particular intended use.

[00124] The oligonucleotide components of reactions of the invention are generally in excess of the number of target nucleic acid sequence to be amplified. They can be provided at about or at least about any of the following: 10, 10^2, 10^4, 10^6, 10^8, 10^{10}, 10^{12} times the amount of target nucleic acid. Promoter primers, splice templates, ligation splint oligos, blocker sequence oligos, strand-displacement primers, and the like, can each be

provided at about or at least about any of the following concentrations: 50 nM, 100 nM, 500 nM, 1000 nM, 2500 nM, 5000 nM, or 10,000 nM, but higher or lower concentrations can also be used. By way of example, but not of limitation, a concentration of one or more oligonucleotides may be desirable for production of one or more target nucleic acid sequences that are used in another application or process. The invention is not limited to a particular concentration of an oligonucleotide, so long as the concentration is effective in a particular method of the invention.

[00125] In some embodiments, the foregoing components are added simultaneously at the initiation of the process. In other embodiments, components are added in any order prior to or after appropriate time points during the process, as required and/or permitted by the reaction. Such time points can readily be identified by a person of skill in the art. The enzymes used for nucleic acid reactions according to the methods of the present invention are generally added to the reaction mixture following a step for denaturation of a double-stranded target nucleic acid in or from a sample, and/or following hybridization of primers and/or oligos of a reaction to a denatured double-stranded or single-stranded target nucleic acid, as determined by their thermal stability and/or other considerations known to the person of skill in the art.

[00126] The reactions can be stopped at various time points, and resumed at a later time. The time points can readily be identified by a person of skill in the art. Methods for stopping the reactions are known in the art, including, for example, cooling the reaction mixture to a temperature that inhibits enzyme activity. Methods for resuming the reactions are also known in the art, including, for example, raising the temperature of the reaction mixture to a temperature that permits enzyme activity. In some embodiments, one or more of the components of the reactions is replenished prior to, at, or following the resumption of the reactions. Alternatively, the reaction can be allowed to proceed (i.e., from start to finish) without interruption.

11. Detection and Identification of Transcription Products or of Compositions Obtained or Derived Therefrom

[00127] In some embodiments, the detection of the product is indicative of the presence of the target sequence. Quantitative analysis, including analysis in real time, can also be performed in some embodiments. Direct and indirect detection methods (including quantification) are well known in the art. For example, by comparing the amount of product amplified from a test sample containing an unknown amount of a polynucleotide

containing a target sequence to the product of a reference sample that has a known quantity of a polynucleotide that contains the target sequence, the amount of target sequence in the test sample can be determined. The methods of the present invention can also be extended to analysis of sequence alterations and sequencing of the target nucleic acid. The amplified nucleic acid can be sequenced using any suitable procedure. Many such procedures are known. Preferred forms of sequencing for use with amplified sequences produced from some embodiments are nanosequencing methods described by Jalanko et al., Clinical Chemistry 38:39-43, 1992; Nikiforov et al., Nucleic Acids Research 22:4167-4175, 1994; and Kobayashi et al., Molecular and Cellular Probes 9:175-182, 1995, and primer extension sequencing, as described in PCT Application WO 97/20948, all of which references are included herein by reference. Further, detection could be effected by, for example, examination of translation products from RNA products.

B. Methods for Obtaining a ssDNA Comprising a Target Sequence

1. General Aspects and Methods for Obtaining a Target Sequence

[00128] An initial step in obtaining a target sequence is rendering the target nucleic acid single-stranded. If the target nucleic acid is a double-stranded DNA (dsDNA), the initial step is target denaturation. The denaturation step may be thermal denaturation or any other method known in the art, such as alkali treatment.

[00129] In some embodiments of the invention in which the target nucleic acid in a sample is DNA, the ssDNA target sequence comprises either ssDNA that is present in a biological sample or ssDNA that is obtained by denaturation of dsDNA in the sample.

[00130] In other embodiments, the ssDNA target sequence comprises ssDNA that is obtained as a result of a "primer extension reaction," meaning an *in vitro* or *in vivo* DNA polymerization reaction using either ssDNA or denatured dsDNA that is present in the sample as a template and an oligonucleotide as a primer under DNA polymerization reaction conditions. A "primer" is an oligonucleotide (oligo), generally with a free 3'-OH group, for which at least the 3'-portion of the oligo is complementary to a portion of the template and which oligo "binds" (or "complexes" or "anneals" or "hybridizes"), by hydrogen bonding and other molecular forces, to the template to give a primer/template complex for initiation of synthesis by a DNA polymerase, and which is extended (i.e., "primer extended") by the addition of covalently bonded bases linked at its 3'-end which are complementary to the template in the process of DNA synthesis. The result is a primer

extension product. Virtually all DNA polymerases (including reverse transcriptases) that are known require complexing of an oligonucleotide to a single-stranded template ("priming") to initiate DNA synthesis, whereas RNA replication and transcription (copying of RNA from DNA) generally do not require a primer.

[00131] In some embodiments, the target nucleic acid in the sample or the primer extension product, or both, are made into smaller DNA fragments by methods known in the art in order to generate a DNA target sequence. In some embodiments using samples containing DNA target nucleic acids, a ssDNA target sequence is obtained using a strand displacement method, such as but not limited to, a methods described in PCT Patent Publication Nos. WO 02/16639; WO 00/56877; and AU 00/29742; of Takara Shuzo Company, Kyoto, Japan; U.S. Patent Nos. 5,523,204; 5,536,649; 5,624,825; 5,631,147; 5,648,211; 5,733,752; 5,744,311; 5,756,702; and 5,916,779 of Becton Dickinson and Company; U.S. Patent Nos. 6,238,868; 6,309,833; and 6,326,173 of Nanogen/ Becton Dickinson Partnership; U.S. Patent Nos. 5,849,547; 5,874,260; and 6,218,151 of Bio Merieux; U.S. Patent Nos. 5,786,183; 6,087,133; and 6,214,587 of Gen-Probe, Inc.; U.S. Patent No. 6,063,604 of Wick et al.; U.S. Patent No. 6,251,639 of Kurn; U.S. Patent No. 6,410,278; and PCT Publication No. WO 00/28082 of Eiken Kagaku Kabushiki Kaishi, Tokyo, Japan; U.S. Patent Nos. 5,591,609; 5,614,389; 5,773,733; 5,834,202; and 6,448,017 of Auerbach; and U.S. Patent Nos. 6,124,120; and 6,280,949 of Lizardi, all of which are incorporated herein by reference. In still other embodiments, the ssDNA target sequence is obtained from a rolling circle replication reaction. The 3'-end of the DNA target sequence can be defined, if it need be defined, by using any suitable method known in the art, such as, but not limited to, a method discussed in the section herein entitled "Methods for Defining the 5'- and 3'-Ends of Target Sequences That Comprise Only a Portion of a Larger RNA or DNA Target Nucleic Acid."

[00132] If the target nucleic acid is RNA, the initial step for obtaining a target sequence comprises synthesis of a single-stranded first-strand cDNA by reverse transcription of the RNA target, meaning an *in vitro* reaction that utilizes an RNA present in a sample as a template and a nucleic acid oligonucleotide that is complementary to at least a portion of a sequence of the RNA template as a primer in order to synthesize ssDNA using an RNA-dependent DNA polymerase (i.e., reverse transcriptase) under reaction conditions. Techniques for the synthesis of cDNA from RNA are known in the art.

[00133] In some embodiments, a first-strand cDNA for use in methods of the invention is synthesized *in situ* in cells or tissue in a tissue section using methods similar to those described in U.S. Patent Nos. 5,168,038; 5,021,335; and 5,514,545, which are incorporated herein by reference. Thus, the first-strand cDNA is synthesized by contacting the cells or tissue in the tissue section under hybridizing conditions with a primer, wherein the primer hybridizes to one or more target sequences in the cell or tissue.

[00134] The present invention comprises a method for making a transcription substrate comprising a circular sense promoter-containing first-strand cDNA that is complementary to a target sequence comprising a target nucleic acid in cells or tissue in a tissue section, the method comprising:

[00135] (a) contacting the cells or tissue in the tissue section under hybridizing conditions with a sense promoter primer, the sense promoter primer comprising (i) a 5'-phosphorylated portion comprising a sense transcription promoter for an RNA polymerase that can synthesize RNA using this promoter in a transcription substrate, and (ii) a 3'-end that is complementary to the target sequence comprising the target nucleic acid;

[00136] (b) contacting the cells or tissue containing the sense promoter primer in the tissue section with a reverse transcriptase under reverse transcription conditions so as to obtain a first-strand cDNA that is complementary to the target sequence;

[00137] (c) obtaining linear sense promoter-containing first-strand cDNA;

[00138] (d) ligating the linear sense promoter-containing first-strand cDNA so as to obtain circular sense promoter-containing first-strand cDNA;

[00139] (e) annealing an anti-sense promoter oligo to the circular sense promoter-containing first-strand cDNA so as to obtain a circular transcription substrate.

[00140] The circular transcription substrate in the cells or tissues can be incubated with an RNA polymerase that uses the promoter under transcription conditions, wherein a transcription product is obtained.

[00141] A primer can have a sequence that is complementary to a specific known sequence in the RNA target in a sample, or a primer can have a sequence comprising a mixture of all possible or many possible sequences, such as, but not limited to, a primer comprising a random hexamer priming sequence. Random primer sequences can be made using an oligonucleotide synthesizer by including nucleotide reagents that are complementary to each of the four canonical bases (i.e., all four nucleotides) during the chemical synthesis of each nucleotide position of the oligonucleotide that is complementary

to the target sequence. In embodiments of the invention using samples containing mRNA targets, the ssDNA target sequence comprises first-strand cDNA that is made by reverse transcription of the mRNA using an oligonucleotide primer comprising either a specific sequence which is complementary to a known sequence of a specific mRNA or, if the mRNA has a poly(A) tail at its 3'-end, an oligo(dT) primer or an oligo(dT) anchor primer. In other embodiments of the invention, a sense promoter primer is used, which serves both to prime synthesis of the first-strand cDNA target sequence and to join a sense transcription promoter to the target sequence.

2. Methods for Defining the 5'- and 3'-Ends of Target Sequences That Comprise Only a Portion of a Larger RNA or DNA Target Nucleic Acid

[00142] When a method of the invention is used to obtain a transcription product corresponding to the complete sequence(s) of one or a multitude of nucleic acid molecules, such as, but not limited to the complete sequences (excluding the cap structure) of substantially all polyadenylated mRNA molecules in a sample, it is not necessary to devise methods to define the 5'- and 3'-ends of the sequences. However, if a method of the invention is used to obtain a transcription product corresponding to a target sequence that comprises only a portion of a larger RNA or DNA nucleic acid in a sample, then methods are needed to delimit the target sequence that becomes the transcription template.

[00143] There are two general approaches for delimiting the ends of the target sequence that becomes the transcription template sequence. In the first direct approach, methods are used to determine the size and end sequences of a target nucleic acid molecule or molecules present in the sample itself. In the second indirect approach, instead of changing the size and end sequences of the target nucleic acid molecules present in a sample, methods are used to determine the size and end sequences of one or more first-strand cDNA molecules that is/are synthesesized by reverse transcription or primer extension, respectively, of RNA or of at least one strand of DNA in a sample.

[00144] With respect to the direct approach, a number of methods are known in the art for cleaving a nucleic acid molecule at or near a specific sequence, and any of the methods which delimit the size and end sequences of a target nucleic acid for an application of the present invention can be used. By way of example, but not of limitation, a DNA in a sample comprising a dsDNA molecule or a ssDNA molecule to which an appropriate complementary DNA oligo is annealed can be digested with a restriction endonuclease, provided a restriction site that would provide a suitable 5'-end and/or 3'-end sequence is

present. Alternatively, one or more DNA oligonucleotides having a double-stranded segment that contains a *Fok*I restriction enzyme site and a single-stranded segment that binds to the desired cleavage site on a first-strand cDNA can be used. As is well known in the art, this type of oligonucleotide can be used with the restriction enzyme *Fok*I to cut a single-stranded DNA at almost any desired sequence (Szybalski, W., Gene 40:169-173, 1985; Podhajska A. J. and Szybalski W., Gene 40:175, 1985, incorporated herein by reference).

[00145] By way of further example, but not of limitation, a ssRNA target nucleic acid present in a sample can be cleaved using a ribonuclease H in regions to which complementary oligonucleotides comprising at least three-to-four deoxynucleotides are annealed. Alternatively, a linear DNA oligonucleotide can be annealed to an RNA in a sample at a location that encodes a recognition site of a restriction enzyme that can cut RNA:DNA heteroduplexes. Cutting the target RNA:DNA oligo with the enzyme will then generate a defined end. Alternatively, an RNA or DNA oligo or polynucleotide with a sequence complementary to the region of an RNA target sequence that is intended to become a transcription substrate can be annealed to the RNA and the sequences of the RNA to which the oligo or polynucleotide is not annealed can be digested using a single-strand-specific ribonuclease, such as RNase A or RNase T1. Still further, either RNA or DNA nucleic acids of known sequence can be cleaved at specific sites using a 5'-nuclease or Cleavase™ enzyme and specific oligonucleotides, as described by Kwiatkowski, et al., (Molecular Diagnosis 4:353-364, 1999) and in U.S. Patent No. 6,001,567 and related patents assigned to Third Wave Technologies (Madison, WI, USA), which are incorporated herein by reference.

[00146] In general, with respect to the second indirect approach, the 5'-end of the primer that is used for reverse transcription of RNA in a sample or for primer extension of at least one strand of DNA in a sample defines the 5'-end of the first-strand cDNA target sequence. Thus, a sample target nucleic acid that is reverse transcribed or primer extended to make a first-strand cDNA target sequence need not have a defined 3'-end.

[00147] In order to generate a defined 3'-end on a first-strand cDNA (i.e., corresponding to the 5'-end of the target sequence), a number of methods can be used to obtain a target sequence of the present invention. By way of example, but not of limitation, if a specific sequence is present in the first-strand cDNA that corresponds to a restriction endonuclease site that would provide a suitable 3'-end sequence, a complementary DNA

oligo can be annealed to this sequence and the site can be cleaved with the restriction enzyme. The complementary DNA oligo used to provide the double-stranded restriction site can optionally have a 2', 3'-dideoxynucleotide or another terminator nucleotide at its 3'-end so that it cannot be extended by a DNA polymerase. Alternatively, the 3'-end of the target sequence can be defined using a DNA oligonucleotide having a double-stranded segment that contains a *Fok*I restriction enzyme site and a single-stranded segment that binds to the desired cleavage site on a first-strand cDNA (Szybalski, W., Gene 40:169-173, 1985; Podhajska A. J. and Szybalski W., Gene 40:175, 1985). Still further, a 5'-nuclease can be used to cleave a first-strand cDNA at a defined 3'-end as discussed above.

[00148] In addition to the above methods, the 3'-end of a first-strand cDNA can also be limited by other methods. A preferred method of the invention is to use a "blocking oligo" or a "blocker sequence," as disclosed by Laney, et al. in U.S. Patent No. 5,679,512, and by Kurn in U.S. Patent No. 6,251,639, both of which are incorporated herein by reference. The "blocker sequence" or "blocker oligo" is a polynucleotide, which is usually a synthetic polynucleotide that is single-stranded and comprises a sequence that is hybridizable, and preferably complementary, to a segment of target nucleic acid, wherein the blocking oligo anneals to the target nucleic acid so as to block further primer extension of the 3'-end of first-strand cDNA at a desired position. Some embodiments of strand displacement methods of the present invention for obtaining a ssDNA target sequence comprise use of a blocking oligo. The blocking oligo comprises nucleotides that bind to the target nucleic acid with an affinity, preferably a high affinity, such that the blocker sequence resists displacement by DNA polymerase in the course of primer extension, in preferably more than about 30%, more preferably more than about 50%, even more preferably more than about 75%, and most preferably more than about 90%, of primer extension events. The length and composition of the blocker polynucleotide should be such that excessive random non-specific hybridization is avoided under the conditions of the methods of the present invention. The length of the blocker polynucleotide is preferably from about 3 to about 30 nucleotides, more preferably from about 5 to about 25 nucleotides, even more preferably from about 8 to about 20 nucleotides, and most preferably from about 10 to about 15 nucleotides. In other embodiments, the blocker polynucleotide is at least about any of the following: 3, 5, 8, 10, 15; and less than about any of the following: 20, 25, 30, 35. It is understood that the length can be greater or less as appropriate under the reaction conditions of the methods of this invention. The

complementarity of the blocker polynucleotide is preferably at least about 25%, more preferably at least about 50%, even more preferably at least about 75%, and most preferably at least about 90%, to its intended binding sequence on the target nucleic acid. In some embodiments, the blocker sequence that hybridizes to a DNA target nucleic acid is attached to the DNA such that displacement of the blocker sequence by the polymerase that affects primer extension is substantially, or at least sufficiently, inhibited. Suitable methods for achieving such attachment include techniques known in the art, such as using a cytosine analog that contains a G-clamp heterocycle modification as described by Flanagan et al., (Proc. Natl. Acad. Sci. USA 96:3513-3518, 1999); and locked nucleic acids as described, e.g., by Kumar et al., (Bioorg. Med. Chem. Lett. 8:2219-2222, 1998; and by Wahlestedt et al. (Proc. Natl. Acad. Sci. USA 97:5633-5638, 2000), all of which are incorporated herein by reference. Other suitable methods include using, where appropriate, sequences with a high GC content and/or cross-linking. Any of these methods for obtaining enhanced attachment may be used alone or in combination. Alternatively, a molecule comprising a peptide nucleic acid ("PNA") can be used.

[00149] Still further, another method that can be used to limit the 3'-end of a first-strand cDNA is to use a thermocycler with short DNA synthesis elongation cycles during reverse transcription or primer extension to synthesize first-strand cDNA. The length of the primer extension product can be somewhat controlled by the length of the DNA synthesis cycle. Conditions can be determined to define an approximate chain length of first-strand cDNA by controlling the temperature and time interval of DNA synthesis before denaturing the growing first-strand cDNA from the template by raising the temperature.

[00150] Further, the 3'-end of a first-strand cDNA that is to become the template sequence for a transcription reaction can be defined by first amplifying the target nucleic acid sequence using any suitable amplification method that delimits the end sequence. By way of example, but not of limitation, it can be prepared using PCR, RT-PCR, NASBA, TMA, 3SR, Ligation Chain Reaction (LCR), Linked Linear Amplification (BioRad), SDA, RCA, ICAN™ (Takara: Sagawa et al. in PCT Patent Publication No. WO 02/16639; and in PCT Patent Publications Nos. WO 00/56877 and AU 00/29742; or a strand-displacement method of Kurn (U.S. Patent No. 6,251,639), all of which are incorporated herein by reference.

[00151] If a 3'-end of a target sequence need not be at an exact location, and can be random or imprecise, which is the case in some embodiments of the invention, there are a number of other methods that can be used for making smaller fragments of a DNA molecule, whether for a target nucleic acid, a target sequence, or otherwise. By way of example, but not of limitation, a target nucleic acid can be fragmented by physical means, such as by movement in and out of a syringe needle or other orifice or by sonication, preferably with subsequent end repair, such as using a T4 DNA polymerase or a kit, such as the End-It™ DNA End Repair Kit (Epicentre Technologies, Madison, WI, USA). Still another method that can be used is to incorporate dUMP randomly into the first-strand cDNA during reverse transcription or primer extension by using dUTP in place of a portion of the TTP in the reaction. The dUMP will be incorporated randomly in place of TMP at a frequency based on the ratio of dUTP to TTP. Then, the first-strand cDNA can be cleaved at sites of dUMP incorporation by treatment (e.g., see U.S. Patent No. 6,048,696, incorporated herein by reference) with uracil-N-glycosylase (UNG) and endonuclease IV (endo IV), which are available from Epicentre Technologies (Madison, WI, USA). UNG hydrolyzes the N-glycosidic bond between the deoxyribose sugar and uracil in single- and double-stranded DNA that contains uracil in place of thymidine. It has no activity on uracil residues in RNA or on dUTP. Endo IV cleaves the phosphodiester linkage at the abasic site. It may be useful to use a thermolabile UNG (e.g., HK™-UNG from Epicentre Technologies, Madison, Wisconsin, USA) for some applications. (Also, incorporation of dUMP at specific sites within a synthetic oligonucleotide or, for example, within a promoter primer of the invention between the 3'-target-sequence-complementary portion and the promoter sequence, introduces specific cleavage sites which can be used at any time to cleave a resulting nucleic acid which contains the site by treatment with UNG and endo IV.) Still further, in some cases, the 3'-end of a first-strand cDNA can be defined by treatment with exonuclease III (Henikoff, S., Gene 28:351, 1984). In still other cases, the 3'-end of a first-strand cDNA that is annealed to a DNA target nucleic acid can be incubated with T4 DNA polymerase or unmodified T7 DNA polymerase (Epicentre Technologies, Madison, WI) in the absence or the presence of dNTPs in the reaction; these enzymes have the 3'-to-5' exonuclease activity in the absence of dNTPs, but the polymerase activity predominates in the presence of dNTPs. These are only some of the methods that can be used to define the 3'-ends of a first-strand cDNA, and the invention is not limited to these methods, which are presented only as examples.

C. Methods for Obtaining a Transcription Substrate

1. Introduction

[00152] In a first general embodiment, first-strand cDNA is obtained by reverse transcription or primer extension using a sense promoter primer comprising a sense transcription promoter in its 5'-portion and a sequence complementary to the target sequence at its 3'-end as a primer and a target nucleic acid sequence as a template. A circular transcription substrate is obtained by ligating the first-strand cDNA to obtain circular sense promoter-containing first-strand cDNA and then annealing an anti-sense promoter oligo to the sense promoter sequence. In another embodiment, a linear transcription substrate having the sense promoter 3'-of the target sequence is obtained by linearizing circular sense promoter-containing first-strand cDNA (e.g., without limiting the invention, using uracil-N-glycosylase and endonuclease IV) and then a circular transcription substrate is obtained by annealing of the anti-sense promoter oligo.

[00153] In still another embodiment, a transcription substrate comprising second-strand cDNA is obtained by:

[00154] (a) synthesizing linear first-strand cDNA using a reverse transcriptase or a DNA polymerase and an anti-sense promoter primer comprising an anti-sense transcription promoter in its 5'-portion and a sequence complementary to the target sequence at its 3'-end;

[00155] (b) ligating the resulting linear first-strand cDNA to obtain a circular first-strand cDNA having an anti-sense transcription promoter;

[00156] (c) obtaining a linear sense-promoter-containing second-strand cDNA by DNA synthesis, preferably rolling circle replication DNA synthesis, using the circular first-strand cDNA as a template, a primer with a 3'-end complementary to a sequence on first-strand cDNA and a DNA polymerase, preferably a strand-displacing DNA polymerase; and

[00157] (d) annealing an anti-sense promoter oligo to obtain a linear transcription substrate. If the second-strand cDNA is obtained by rolling circle replication the linear transcription substrate obtained is a concatemeric linear transcription substrate. In this embodiment, the transcription product of the transcription substrate comprises an anti-sense transcription product.

[00158] The embodiments of processes for obtaining a transcription substrate for methods of the invention described above are provided only as examples, and are not intended to limit the present invention. The description above and herein will reveal and

make evident to those with knowledge in the art numerous other embodiments of methods and processes for obtaining a transcription substrate for use in making a transcription product corresponding to a target nucleic acid sequence in a method of the invention, and the invention includes all of those methods and processes for obtaining a transcription substrate for use in the methods.

[00159] In embodiments in which the target sequences comprise mRNA, whether of a single species of mRNA or all of the mRNA in a particular sample, the transcription products can subsequently be used for a variety of applications. By way of example, but not of limitation, transcription products can be used for *in vitro* or *in vivo* translation, for use as RNAi to silence one or more genes *in vivo,* for spotting on a surface to make expression arrays or microarrays, or for making hybridization probes for arrays or microarrays for gene expression profiling or other uses. In still other embodiments, methods of the invention can be used to make first-strand cDNA from mRNA, which in turn can be used for techniques such as random amplification of cDNA ends (RACE) or to make hybridization probes.

[00160] A "ligation splint" or a "ligation splint oligo" is an oligo that is used to provide an annealing site or a "ligation template" for joining two ends of one nucleic acid (i.e., "intramolecular joining") or two ends of two nucleic acids (i.e., "intermolecular joining") using a ligase or another enzyme with ligase activity. The ligation splint holds the ends adjacent to each other and "creates a ligation junction" between the 5'-phosphorylated and a 3'-hydroxylated ends that are to be ligated. For example, when a ligation splint oligo is used to join a sense promoter ligation oligo to the 3'-end of a first-strand cDNA comprising a target sequence, the ligation splint oligo has a sequence complementary to the 3'-end of the target sequence, including a "tailed" target sequence, if any, and a second adjacent sequence that is complementary to the 5'-end of a the 5'-phosphorylated promoter ligation oligo. Ligases that can be used to ligate suitable ends that are annealed to a ligation splint comprising DNA include, but are not limited to, Ampligase® DNA Ligase (EPICENTRE Technologies, Madison, WI), Tth DNA ligase, Tfl DNA ligase, Tsc DNA ligase (Prokaria, Ltd., Reykjavik, Iceland), or T4 DNA ligase. These ligases can be used for both intermolecular and intramolecular ligations when a ligation splint comprising DNA is used to bring the respect ends adjacent. If a ligation splint comprising RNA is used, T4 DNA ligase can be used to join the ends that are annealed to the ligation splint. In some embodiments, a ligase that catalyzes non-homologous intramolecular ligation of a 5'-

phosphorylated end with a 3'-hydroxyl end can be used in methods of the invention for circularization of single-stranded DNA without a ligation splint. By way of example, but not of limitation, a ligation splint is not required for ligation of a ssDNA using ThermoPhage™ RNA Ligase II (Prokaria, Ltd., Reykjavik, Iceland).

[00161] In some embodiments, remaining linear nucleic acids, such as, but not limited to, ligation splint oligos, are removed during the reaction using the gene 6 exonuclease of phage T7. This exonuclease digests DNA starting from the 5'-end of a double-stranded structure. It has been used successfully for the generation of single-stranded DNA after PCR amplification (Holloway et al., Nucleic Acids Res. 21:3905-3906, 1993; Nikiforov et al., PCR Methods and Applications 3:285-291, 1994, incorporated herein by reference). The gene 6 exonuclease of phage T7 can be added after ligation, together with the rolling circle DNA polymerase to remove unligated oligos. To protect the product of a strand displacement reaction from degradation, a strand displacement primer for rolling circle replication can contain 3 or 4 phosphorothioate linkages at the 5'-end, to make this molecule resistant to the exonuclease (Nikiforov et al., PCR Methods and Applications 3:285-291, 1994). The exonuclease degrades unprotected linear molecules as they become associated with the rolling circle DNA product. Based on this description, those with knowledge in the art will understand and know other embodiments of the invention in which this process of the invention for removing single-stranded DNA oligos can be used to advantage, and the invention comprises all such embodiments.

[00162] A ligation splint or a ligation splint oligo provides an annealing site or a "ligation template" on which a 5'-phosphorylated end and a 3'-hydroxyl end of one or two different nucleic acids, such as, but not limited to, a promoter ligation oligo and a first-strand cDNA target nucleic acid sequence, can hybridize so as to bring the two ends adjacent to one another in a ligation reaction for joining by a ligase (or a topoisomerase or other enzyme with ligase activity). A ligation splint comprises a sufficient number and composition of nucleotides, and is present in sufficient concentration, so that the complementary sequences of both the 5'- and 3'-ends remain annealed to the ligation splint under ligation conditions so to permit ligation of the ends to occur. Thus, in most embodiments, the ligation splint comprises at least about 4 nucleotides up to about 20 nucleotides, but the invention is not limited to a specific number of nucleotides. An appropriate sequence (and T_m), size, and concentration for a ligation splint can be determined empirically by those with knowledge in the art. In most embodiments of the present invention, it is preferable that the 3'-terminal

nucleotide of a ligation splint is a dideoxynucleotide or another termination nucleotide, so that the ligation splint oligo cannot serve as a primer for polymerases in the reaction. In most embodiments in which there are multiple rounds of transcription of a target nucleic acid sequence, the other nucleotides in a ligation splint oligo comprise deoxynucleotides. However, in some embodiments, the other nucleotides can comprise ribonucleotides and/or purine ribonucleotides and 2'-fluoro-pyrimidine nucleotides, which confer resistance to RNase A-type nucleases. The composition of a ligation splint oligo will also depend on the ligase and ligation conditions used. By way of example, Ampligase® Thermostable Ligase (Epicentre Technologies, Madison, WI, USA) will only ligate the 5'-phosphoryl and 3'-hydroxyl ends of DNA that is annealed to a DNA ligation splint and will not ligate DNA ends annealed to RNA. However, T4 RNA ligase has been reported to efficiently ligate DNA ends that are annealed to an RNA ligaton splint (Faruqui et al., U.S. Patent No. 6,368,801, incorporated herein by reference). Ligation splint oligos can be synthesized on an oligo synthesizer, which is usually preferred, or enzymatically, using methods discussed elsewhere herein.

[00163]

2. Obtaining Transcription Substrates Using a Promoter Primer

a. Methods for Using Promoter Primers and Anti-Sense Promoter Oligos

[00164] A "promoter primer" is a primer, generally with a free 3'-OH group, that comprises a sequence that is complementary to a target sequence at its 3'-end and which encodes a transcription promoter in its 5'-portion. The transcription promoter in the 5'-end portion can be either a "sense" promoter" or an "anti-sense promoter." As defined herein, the promoter sequence of a double-stranded promoter that is operably joined to the 3'-end of the template strand sequence that is transcribed is a "sense promoter sequence" and a promoter primer that comprises this sequence is "a sense promoter primer." The sequence of a double-stranded promoter that is complementary to the sense promoter is defined herein as "an anti-sense promoter" and a promoter primer that comprises this sequence is "an anti-sense promoter primer."

[00165] A sense promoter primer is used in most embodiments of the present invention. If a sense promoter primer is used, a "circular transcription substrate" that comprises a functional double-stranded promoter can be obtained by annealing "an anti-sense promoter oligo" to a molecule that is obtained by primer extension of the sense promoter primer on a

template (which is usually a target nucleic acid) and then ligating the resulting first-strand cDNA to obtain a "circular sense promoter-containing first-strand cDNA." That is, annealing of the anti-sense promoter oligo to the circular sense promoter-containing first-strand cDNA makes a circular transcription substrate. If the circular sense promoter-containing first-strand cDNA is linearized 3'-of the sense promoter sequence therein using methods described elsewhere in the description of the invention, a "linear sense promoter-containing first-strand cDNA" is obtained. A "linear transcription substrate" is obtained by annealing an anti-sense promoter oligo to the linear sense promoter-containing first-strand cDNA.

[00166] An anti-sense promoter oligo is also used in other embodiments of the invention for obtaining a transcription substrate, such as in embodiments wherein the anti-sense promoter oligo is complexed with a linear sense promoter-containing second-strand cDNA to obtain a transcription substrate, as described elsewhere herein.

[00167] An "antisense promoter oligo" as defined herein comprises an oligonucleotide that comprises a sequence for an anti-sense transcription promoter, wherein an RNA polymerase can use the double-stranded complex between the anti-sense promoter oligo and a sense promoter that is joined to the 3'-end of a template sequence to make a transcription product that is complementary to the template under transcription conditions.

[00168] A promoter primer can have a sequence at its 3'-end that is complementary to a specific known sequence in a target nucleic acid. , in which case it is referred to as a "specific-sequence promoter primer." However, other embodiments of promoter primers can also be used in methods of the invention. An "oligo(dT) promoter primer" has an oligo(dT) sequence at its 3'-end, and is used mainly in embodiments of the invention which pertain to mRNA molecules having polyadenylated [i.e., poly(A) tails], although an oligo(dT) promoter primer can also be used in embodiments in which another target nucleic acid is tailed with poly(A) or poly(dA). An "anchored oligo d(T) promoter primer," in addition to having an oligo(dT) sequence in its 3'-portion, also has one (or a small number) of nucleotides 3'-of the oligo(dT) sequence, called "anchor nucleotides," which anneal to the 3'-portion of the mRNA target sequence just prior to the poly(A) sequence. Thus, the anchor nucleotides serve to "anchor" the mRNA-complementary portion of the anchored oligo(dT) promoter primer to the beginning of the protein-coding sequence of the mRNA target sequence. The anchor nucleotides can comprise either a specific base for a specific mRNA or a randomized nucleotide (i.e., synthesized with a mixture of all four nucleotides)

for priming all mRNA molecules in a sample. A "random-sequence promoter primer" has a random sequence, such as, but not limited to a random hexamer sequence or a random octamer sequence, at its 3'-end. In most cases, a random-sequence promoter primer comprises a mixture of primers with all possible sequences (e.g., all possible hexamers) in its target sequence-complementary portion. Random-sequence promoter primers can be made by including all four canonical nucleotide reagents during the chemical synthesis of each of the nucleotide positions of the random sequence (e.g., the hexamer sequence) of the target sequence-complementary portion of the primer. A random-sequence promoter primer can be used in those embodiments of the invention in which it is desired to amplify all target nucleic acid sequences in a sample, or to amplify all target nucleic acid sequences in a random manner, such as for making a library of all target nucleic acid sequences. However, although all sequences may be amplified, use of a random-sequence promoter primer does not necessarily generate only full-length copies of target nucleic acids (e.g., full-length cDNA copies of mRNA molecules from a cell). Thus, embodiments of the invention which use random-sequence promoter primers are usually used when full-length copies of a target nucleic acid sequence are not required, such as, for obtaining hybridization probes for some applications.

[00169] In embodiments of the invention in which a transcription substrate of the invention comprises first-strand cDNA obtained by reverse transcription or primer extension of a promoter primer using a target nucleic acid as a template, the transcription promoter in the promoter primer comprises a sense promoter sequence that is located in the 5'-portion of the promoter primer. Thus, the transcription promoter in the linear first-strand cDNA obtained by reverse transcriptase- or DNA polymerase-catalyzed extension of the promoter primer using the target nucleic acid as a template is not operable as a promoter for transcription of the target sequence since the promoter is not operably joined to the 3'-end of the target sequence. A method of the present invention solves this problem by operably joining the single-stranded sense transcription promoter in the 5'-portion of the linear first-strand cDNA to the 3'-end of the target sequence using a ligase or another joining means, thereby forming a "circular sense promoter-containing first-strand cDNA" that can be used to obtain a circular transcription substrate by annealing of an anti-sense promoter oligo to the sense promoter sequence. The circular transcription substrate can be used to make a transcription product corresponding to the target sequence by transcription using an RNA

polymerase that can bind the single-stranded promoter and transcribe the target sequence joined thereto.

[00170] Thus, in one embodiment of the invention, a promoter primer having a sequence complementary to a target sequence at its 3'-end and a transcription promoter in its 5'-portion is used to obtain a circular transcription substrate of the invention (FIG. 1). After annealing to a target nucleic acid, the promoter primer is used to prime first-strand cDNA synthesis using a DNA polymerase or reverse transcriptase under suitable reaction conditions in order to obtain linear first-strand cDNA.

[00171] The first-strand cDNA is then ligated using a ligase under suitable ligation conditions, or using another joining method, such as, but not limited to a topoisomerase (e.g., see U.S. Patent No. 5,766,891, incorporated herein by reference), under suitable joining conditions, so as to obtain a circular promoter-containing first-strand cDNA. By ligation of the phosphorylated 5'-end of linear first-strand cDNA to its 3'-end, the transcription promoter is joined to the target sequence so that *in vitro* transcription using an RNA polymerase of the invention under transcription conditions will synthesize transcription products corresponding to the target sequence.

[00172] In the example in FIG. 1, the target sequence can comprise target nucleic acid comprising all mRNA molecules in a sample and the transcription products that are made by transcription of the transcription substrate comprise RNA that has essentially the same sequence as sense mRNA. If there is no sequence in the circular transcription substrate that results in termination of transcription (i.e., a transcription terminator sequence), transcription continues around and around the circular transcription substrate multiple times and generates concatemers of sense transcription products (i.e., comprising tandem copies of the same nucleic acid sequence as an mRNA target nucleic acid sequence in the sample), which concatemers are useful for certain applications of the invention.

[00173] In some embodiments of the invention, one or more transcription termination sequences is/are incorporated into the promoter primer between the target sequence-complementary 3'-region and the transcription promoter 5'-portion in order to permit synthesis of single-copy rather than concatemeric sense transcription products. For example, if a transcription termination sequence is present in the promoter primer, the transcription product can correspond in length to a single copy of an mRNA target nucleic acid sequence following *in vitro* transcription of the circular transcription substrate using an RNA polymerase of the invention. Transcription termination sequences are known in the

art and those with knowledge in the art will know how to find information about the sequences, as well as experimental methods for identifying additional termination sequences that can be used. By way of example, but not of limitation, information about transcription termination sequences can be found in a book entitled "RNA Polymerases and the Regulation of Transcription," edited by Reznikoff, W.S., et al., (Elsevier Science Publishing Co., Inc., New York, 1987) and in Section 17 of a book by Miller, J.H. entitled "A Short Course in Bacterial Genetics. A Laboratory Handbook for Escherichia coli and Related Bacteria" (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1992), both incorporated herein by reference.

[00174] In still other embodiments, the circular sense promoter-containing first-strand cDNA obtained by ligation of the first-strand cDNA is linearized prior to use for *in vitro* transcription in order to form a "linear sense promoter-containing first-strand cDNA," which is annealed to an anti-sense promoter oligo to obtain a linear transcription substrate of the invention (Figure 2).

[00175] By way of example, but not of limitation, the circular sense promoter-containing first-strand cDNA can be linearized by treatment with uracil-N-gycosylase ("UNG") and endonuclease IV ("endo IV") (e.g., see methods in U.S. Patent No. 6,048,696, which is incorporated herein by reference) if the promoter primer is synthesized to have a dUMP nucleotide between the target sequence-complementary 3'-region and the transcription promoter in its 5'-portion. However, the use of a promoter primer comprising a dUMP nucleotide can only be used in embodiments of the invention which are performed in a stepwise manner, because the presence of UNG in a continuous reaction would cleave the transcription promoter portion of a promoter primer from the target-complementary portion of the promoter primer, thus destroying the ability of the promoter primer to generate more transcription substrates. There are a number of other methods known in the art for linearizing a circular DNA molecule, which can be used in embodiments of the invention, and those with knowledge in the art will know or know how to find suitable methods for use in the invention. By way of example, but not of limitation, a number of such methods which can be used are described herein in the section entitled "Methods for Defining the 5'- and 3'-Ends of Target Sequences That Comprise Only a Portion of a Larger RNA or DNA Target Nucleic Acid."

[00176] Other embodiments of the invention comprise use of a promoter primer for synthesis of a second-strand cDNA as a transcription substrate of the invention. In those

embodiments, a transcription promoter can be incorporated into the second-strand cDNA by, either (a) synthesizing first-strand cDNA using a promoter primer that has a sequence in its 5'-portion comprising a sequence that is complementary to a sense transcription promoter (i.e., it comprises an anti-sense promoter primer) or, (b) using a promoter primer comprising a sense transcription promoter for synthesis of second-strand cDNA. The promoter sequence used in a promoter primer can be determined based on knowledge of sequences of sense promoters for RNA polymerases of the invention and of the rules of nucleic acid base complementarity and the directionality of DNA and RNA synthesis by DNA and RNA polymerases. By way of example, if a sense transcription promoter is desired in a second-strand cDNA that is made by primer extension of first-strand cDNA, which is in turn made by reverse transcription of mRNA, one can work backwards from having a sense transcription promoter of known sequence in a transcription substrate at the 3'-end of a target nucleic acid sequence that is to be amplified by an RNA polymerase of the invention, in order to determine the appropriate sense or anti-sense sequence (and position of the sequence with respect to the target sequence) that is needed, respectively, for promoter primers that are used for first-strand cDNA synthesis, or for second-strand cDNA synthesis.

[00177] In some embodiments of the invention, more than one promoter can be present on the promoter primer. By way of example, but not of limitation, a promoter primer can encode two promoter sequences, both of which encode sense promoters on first-strand cDNA (e.g., for two different RNA polymerases of the invention). Alternatively, a first promoter sequence of the promoter primer and the resulting first-strand cDNA can comprise a sense promoter and a second promoter sequence can comprise an anti-sense promoter, in which case, the first promoter sequence will be anti-sense and the second promoter sequence will be sense in second-strand cDNA. In embodiments that include additional rounds of transcription by using RNA from the first round to obtain a second transcription substrate for transcription, it is necessary to take into account the fate of the promoter sequences through the subsequent rounds of transcription when designing the reaction.

[00178] In addition to the transcription promoter sequence and the target-complementary sequence, a promoter primer of the invention can also have additional nucleic acid sequences that are 5'-of and/or 3'-of the transcription promoter sequence, but a promoter primer is not required to have such additional other sequences. By way of example, but not

of limitation, a promoter primer can have a transcription initiation site 5'-of the promoter sequence. In some embodiments of the invention, a promoter primer can have one or more transcription termination sequences, one or more sites for DNA cleavage, (such as, but not limited to, a dUMP residue that can be cleaved using uracil-N-glycosylase and endonuclease IV, or other cleavage methods discussed elsewhere herein) to permit controlled linearization of a circular first-strand cDNA that is a transcription substrate, one or more origins ("*ori*'s") of replication (preferably an *ori* for a single-stranded replicon, such as, but not limited to, a phage M13 replicon), a selectable or screenable marker, such as, but not limited to an antibiotic-resistance gene or a beta-galactosidase gene, respectively, or one or more transposon recognition sequences (e.g., OE or ME sequences) that can be recognized and used by a transposase for *in vitro* or *in vivo* transposition, or one or more sites that are recognized by a recombinase (such as, but not limited to, the cre-lox system), and/or other sequences or genetic elements for a particular purpose. After reading the specification of the present invention, those with knowledge in the art will know that a sequence that is 5'-of a functional promoter will be transcribed by an RNA polymerase of the invention, and will therefore know where to position particular additional sequences or genetic elements relative to the promoter sequence in a promoter primer. In some embodiments of the invention, a promoter primer has a 5'-phosphate or is phosphorylated at its 5'-end using an enzyme, such as, but not limited to, a polynucleotide kinase (e.g., T4 PNK), during the processes of a method of the invention. A primary reason for providing a 5'-phosphate group on a promoter primer is to permit ligation of linear first-strand cDNA following reverse transcription or primer extension of a promoter primer on a target sequence in order to obtain a circular promoter-containing first-strand cDNA.

[00179] A number of examples of embodiments that use promoter primers of the invention are described below. The invention comprises all methods for using a sense promoter primer wherein the transcription promoter, in double-stranded form, can be used by an RNA polymerase that can synthesize RNA using a transcription substrate and is not limited to only the example embodiments presented.

[00180] With respect to methods that use a promoter primer, one embodiment of the invention comprises a method for using a sense promoter primer for making a circular transcription substrate for making transcription product corresponding to a target sequence in a target nucleic acid, the method comprising:

[00181] a. obtaining a sense promoter primer for synthesis of a first-strand cDNA, the promoter primer comprising a sequence at its 3'-end that is complementary to the 3'-end of the target sequence that is to be transcribed, and a 5'-end portion comprising a sequence for a sense transcription promoter, and optionally, a phosphate group or a topoisomerase moiety on its 5'-end;

[00182] b. annealing the promoter primer to the target nucleic acid;

[00183] c. primer-extending or the promoter primer annealed to the target nucleic acid with a DNA polymerase under DNA synthesis conditions so as to obtain first-strand cDNA that is complementary to the target sequence or sequences to which the promoter primer was annealed;

[00184] d. optionally, removing the target nucleic acid that is annealed to the first-strand cDNA;

[00185] e. ligating the first-strand cDNA, wherein the 5'-end of the first-strand cDNA is covalently joined to the 3'-end of the first-strand cDNA so as to obtain circular first-strand cDNA, wherein the circular first-strand cDNA comprises circular sense promoter-containing first-strand cDNA; and

[00186] f. annealing an anti-sense promoter oligo to the circular sense promoter-containing first-strand cDNA so as to obtain a circular transcription substrate.

[00187] Another embodiment of the invention comprises a method for obtaining a linear transcription substrate for making a transcription product corresponding to a target sequence in a target nucleic acid, the method comprising:

[00188] a. obtaining a circular sense promoter-containing first-strand cDNA circular transcription substrate by carrying out steps (a) through (e) of the method immediately above;

[00189] b. linearizing the circular sense promoter-containing first-strand cDNA at a site 3'-of the transcription promoter and 5'-of the target-complementary sequence of the sense promoter primer portion of said circular sense promoter-containing first-strand cDNA, wherein a linear sense promoter-containing first-strand is obtained; and

[00190] c. annealing an anti-sense promoter oligo to the linear sense promoter-containing first-strand cDNA so as to obtain a linear transcription substrate.

[00191] A general embodiment of the invention comprises a method for making a transcription product corresponding to a target sequence in a target nucleic acid, the method comprising:

[00192] a. obtaining a transcription substrate, chosen from among a circular transcription substrate and a linear transcription substrate;

[00193] b. contacting the transcription substrate under transcription conditions with an RNA polymerase that recognizes the promoter in the transcription substrate and makes a transcription product therefrom; so as to obtain transcription product; and

[00194] c. obtaining the transcription product.

[00195] The target nucleic acid can be DNA or RNA. By way of example, but not of limitation, a target sequence can comprise a target nucleic acid comprising a single species of mRNA or a target sequence can comprise a target nucleic acid, which can comprise all of the mRNA in a sample.

[00196] Thus, one embodiment of the invention is a method for using a sense promoter primer for making a transcription product corresponding to a target sequence comprising a target nucleic acid comprising mRNA, the method comprising:

[00197] a. obtaining a target nucleic acid comprising mRNA;

[00198] b. obtaining a sense promoter primer comprising a a target-complementary portion at its 3'-end that is complementary to the 3'-end of the target sequence, wherein the target complementary sequence is chosen from among an oligo(dT) sequence, an anchored oligo(dT)X sequence, a target-specific sequence, and a random sequence, and the5'-end optionally comprises a phosphate or topoisomerase moiety;

[00199] c. annealing the promoter primer to the target nucleic acid;

[00200] d. primer-extending the promoter primer annealed to the target nucleic acid with a DNA polymerase under DNA synthesis conditions so as to obtain first-strand cDNA that is complementary to the target sequence or sequences to which the promoter primer was annealed;

[00201] e. optionally, removing the RNA that is annealed to the first-strand cDNA;

[00202] f. ligating the first-strand cDNA, wherein the 5'-end is covalently joined to the 3'-end of the first-strand cDNA so as to obtain circular sense promoter-containing first-strand cDNA;

[00203] g. annealing an anti-sense promoter oligo to the circular sense promoter-containing first-strand cDNA so as to obtain a circular transcription substrate

[00204] h. contacting the circular transcription substrate with an RNA polymerase under transcription conditions so as to obtain transcription product; and

[00205] i. obtaining the transcription product.

[00206] In another embodiment, the circular sense-promoter first-strand cDNA is linearized to obtain linear sense-promoter first-strand cDNA, and then an anti-sense promoter primer is annealed to the sense promoter sequence therein to obtain a linear transcription substrate. Then, the linear transcription substrate is contacted with an RNA polymerase under transcription conditions so as to obtain transcription product, and transcription product is obtained.

[00207] The methods for obtaining a transcription substrate and for making a transcription product corresponding to a target sequence can be performed in a stepwise manner, or, under suitable reaction conditions, they can be performed continuously in a single reaction mixture.

[00208] Thus, one embodiment of the invention comprises a method for obtaining additional rounds of transcription of a target sequence in a target nucleic acid (FIG. 3), the method comprising:

[00209] a. obtaining a transcription product;

[00210] b. obtaining a sense promoter primer comprising a target-complementary portion at its 3'-end that is complementary to the 3'-end of the target sequence and optionally, a phosphate group or a topoisomerase moiety on its 5-end;

[00211] c. annealing the promoter primer to the transcription product;

[00212] d. primer-extending the promoter primer annealed to the transcription product with a DNA polymerase under DNA synthesis conditions so as to obtain first-strand cDNA;

[00213] e. optionally, removing the RNA that is annealed to the first-strand cDNA;

[00214] f. ligating the first-strand cDNA, wherein the 5'-end is covalently joined to the 3'-end of the first-strand cDNA so as to obtain circular sense promoter-containing first-strand cDNA;

[00215] g. annealing an anti-sense promoter oligo to the circular sense promoter-containing first-strand cDNA so as to obtain a circular transcription substrate

[00216] h. contacting the circular transcription substrate with an RNA polymerase under transcription conditions so as to obtain additional transcription product; and

[00217] i. obtaining the additional transcription product.

[00218] In another embodiment, the circular sense-promoter first-strand cDNA is linearized to obtain linear sense-promoter first-strand cDNA, and then an anti-sense promoter primer is annealed to the sense promoter sequence therein to obtain a linear transcription substrate. Then, the linear transcription substrate is contacted with an RNA polymerase under transcription conditions so as to obtain transcription product, and transcription product is obtained.

[00219] Different embodiments of methods of the invention can also be used to make a transcription product corresponding to target sequences that are internal to a target nucleic acid sequence. By way of example, some embodiments can be used to make a transcription product corresponding to sequences, such as, but not limited to, wild-type or mutated sequence in genomic DNA. In these embodiments, one or more processes, such as, but not limited to, annealing a blocking oligo to the target nucleic acid, are required to limit the 3'-end of the target sequence that is transcribed, as is discussed elsewhere herein. In general, the target nucleic acid must be single-stranded for use in a method of the invention. Thus, a double-stranded nucleic acid must be denatured.

[00220] Thus, one embodiment of the invention for using a promoter primer is a method for making a transcription product corresponding to a target sequence that comprises only a portion of a target nucleic acid comprising single-stranded DNA or RNA, the method comprising:

[00221] a. obtaining a target nucleic acid comprising single-stranded DNA or RNA;

[00222] b. obtaining a sense promoter primer comprising a target-complementary portion at its 3'-end that is complementary to the 3'-end of the target sequence and optionally, a phosphate group or a topoisomerase moiety on its 5-end;

[00223] c. obtaining a blocking oligo, the blocking oligo comprising a sequence that anneals tightly to a sequence on the target nucleic acid so as to delimit the 3'-end of a primer extension product of the promoter primer using the target nucleic acid as a template, wherein the blocking oligo is not displaced by the primer extension product, and wherein the blocking oligo is not itself capable of being primer extended by a DNA polymerase;

[00224] d. annealing the promoter primer and the blocking oligo to the target nucleic acid;

[00225] d. primer-extending the promoter primer annealed to the target nucleic acid with a DNA polymerase under DNA synthesis conditions so as to obtain first-strand cDNA;

[00226] f. optionally, removing the target nucleic acid that is annealed to the first-strand cDNA;

[00227] g. ligating the first-strand cDNA, wherein the 5'-end is covalently joined to the 3'-end of the first-strand cDNA so as to obtain circular sense promoter-containing first-strand cDNA;

[00228] h. optionally, linearizing the circular sense promoter-containing first-strand cDNA at a site that is 3'-of the promoter sequence and 5'-of the target-complementary portion of the promoter primer in circular sense promoter-containing first-strand cDNA so as to obtain linear sense promoter-containing first-strand cDNA;

[00229] g. annealing an anti-sense promoter oligo to the circular sense promoter-containing first-strand cDNA so as to obtain a circular transcription substrate; or annealing an anti-sense promoter oligo to the linear sense promoter-containing first-strand cDNA so as to obtain a linear transcription substrate;

[00230] h. contacting the circular transcription substrate or the linear transcription substrate with an RNA polymerase under transcription conditions so as to obtain transcription product; and

[00231] i. obtaining the transcription product.

[00232] k. optionally, repeating steps b through k to obtain additional rounds of transcription to obtain transcription products.

[00233] In still other embodiments of the invention, the circular first-strand cDNA comprising an anti-sense promoter is used as a template for DNA synthesis using a strand-displacing DNA polymerase and at least one strand displacement primer, and in some embodiments, multiple strand displacement primers. A "strand displacement primer," as used herein, is an oligonucleotide or polynucleotide that can be primer extended by a strand-displacing DNA polymerase of the invention, wherein strand displacement DNA synthesis occurs. In general, strand displacement is more a property of the DNA polymerase and the reaction conditions used than of the primer. Thus, the composition and properties of a strand displacement primer can vary greatly. For example, in some embodiments of the invention, a strand displacement primer can be an oligonucleotide that is hybridizable to a circular DNA template, wherein the DNA synthesis product resulting

from rolling circle replication by a strand displacing DNA polymerase results in displacement of the strand displacement primer extension product, resulting in tandem complementary ssDNA copies of circular template. In other embodiments, it is preferred that the strand displacement primer has a 3'-portion that is complementary to a sequence in a circular DNA template and a 5'-portion that is non-complementary, and therefore has a "flap;" the protruding flap appears to facilitate displacement of the primer in some cases. When strand displacement is carried out on linear DNA templates, strand displacement primers can be designed to have particular nucleotide compositions and/or structures, and additional methods and reaction components can be used in order to facilitate strand displacement by liberating the strand displacement primer from the template.

[00234] A strand-displacing DNA polymerase of the invention can be any DNA polymerase that results in strand displacement. Preferred strand-displacing DNA polymerases of the present invention lack 5'-exonuclease activity, including structure-dependent 5'-nuclease activity. Preferred strand-displacing DNA polymerases comprise rBst DNA polymerase large fragment, also called "IsoTherm™ DNA Polymerase" (Epicentre Technologies, Madison, Wisconsin, USA), Bca DNA Polymerase (TAKARA Shuzo Company, Kyoto, Japan), RepliPHI™ DNA Polymerase (Epicentre Technologies, Madison, Wisconsin, USA), ϕ29 DNA polymerase (U.S. Patent Nos. 5,198,543 and 5,001,050, incorporated herein by reference), SequiTherm™ DNA Polymerase (Epicentre Technologies, Madison, Wisconsin, USA), MMLV reverse transcriptase, and Sequenase® DNA Polymerase (USB, Cleveland, Ohio, USA). In these embodiments, a strand displacement primer is used to prime second-strand DNA synthesis using circular first-strand cDNA as a template. Once DNA synthesis has proceeded completely around the circular first-strand cDNA template, the second-strand cDNA is displaced so that the displaced single-stranded second DNA strand is released into the reaction medium. Since a transcription promoter is present at least once in every round of DNA synthesis of the circular first-strand cDNA template, this released single-stranded "sense-promoter-containing second-strand cDNA" can be used to obtain a linear transcription substrate after annealing of an anti-sense promoter oligo. As the second-strand cDNA continues to grow, longer and longer concatemers of the anti-sense RNA are formed. The linear transcription substrate obtained can be used by an RNA polymerase of the invention under transcription conditions to make an anti-sense transcription product with respect to the target nucleic acid sequence. Thus, this differs from previous embodiments that used sense promoter

primers to make sense transcription products using first-strand cDNA as a template. Anti-sense RNA can be used for many applications, such as, but not limited to, for use as probes for nucleic acid arrays or microarrays. As the second-strand cDNA continues to grow, longer and longer concatemers of the anti-sense RNA are formed.

[00235] As discussed above, some embodiments obtain a transcription template for making a transcription product corresponding to a target sequence in a target nucleic acid by first obtaining a circular first-strand cDNA comprising an anti-sense transcription promoter, wherein the circular first-strand cDNA is then used as a template for strand displacement DNA synthesis of the linear sense promoter-containing second-strand cDNA by a strand-displacing DNA polymerase. A linear transcription substrate is obtained by annealing an anti-sense promoter oligo to the linear sense promoter-containing second-strand cDNA.

[00236] Thus, one embodiment of the invention comprises a method for making a transcription product corresponding to a target sequence in a target nucleic acid (**FIG. 6**), the method comprising:

[00237] a. obtaining a target nucleic acid comprising single-stranded DNA or RNA;

[00238] b. obtaining an anti-sense promoter primer comprising a 3'-end portion that is complementary to a target sequence and optionally, a phosphate group or a topoisomerase moiety on its 5-end;

[00239] c. optionally, obtaining a blocking oligo, the blocking oligo comprising a sequence that anneals tightly to a sequence on the target nucleic acid so as to delimit the 3'-end of a primer extension product of the promoter primer using the target nucleic acid as a template, wherein the blocking oligo is not displaced by the primer extension product, and wherein the blocking oligo is not itself capable of being primer extended by a DNA polymerase;

[00240] d. annealing the promoter primer and, optionally, the blocking oligo to the target nucleic acid;

[00241] e. primer-extending or the promoter primer annealed to the target nucleic acid with a DNA polymerase under DNA synthesis conditions so as to obtain first-strand cDNA;

[00242] f. optionally, removing the target nucleic acid that is annealed to the first-strand cDNA;

[00243] g. ligating the linear first-strand cDNA, wherein the 5'-end is covalently attached to the 3'-end of the first-strand cDNA so as to obtain circular first-strand cDNA;

[00244] h. obtaining a strand-displacement primer;

[00245] i. annealing the strand displacement primer to the circular first-strand cDNA;

[00246] j. obtaining a strand-displacing DNA polymerase;

[00247] k. contacting the circular first-strand cDNA to which the strand displacement primer is annealed with a strand-displacing DNA polymerase under DNA synthesis conditions so as to obtain linear sense promoter-containing second-strand cDNA;

[00248] l. obtaining the linear sense promoter-containing second-strand cDNA;

[00249] m. annealing an anti-sense promoter oligo to the linear sense promoter-containing second-strand cDNA and obtaining a linear second-strand transcription substrate;

[00250] n. obtaining an RNA polymerase that transcribes the linear second-strand transcription substrate using the promoter under transcription conditions;

[00251] o. contacting the linear second-strand transcription template with the RNA polymerase under transcription conditions so as to obtain anti-sense transcription products; and

[00252] p. obtaining the anti-sense transcription products; and

[00253] q. optionally, repeating steps b through q to obtain additional rounds of transcription of anti-sense transcription products.

b. Composition of Promoter Primers and Anti-Sense Promoter Oligos of the Invention

[00254] In preferred embodiments of the invention, a promoter primer comprises DNA nucleotides. A promoter primer can also comprise one or more modified nucleotides for a particular purpose. By way of example, but not of limitation, a promoter primer can comprise one or more dUMP nucleotides 3'-of a sense transcription promoter and 5'-of the sequence that is complementary to a target nucleic acid sequence, which provides a site for linearizing a sense promoter-containing first-strand cDNA (prior to annealing an anti-sense promoter oligo to obtain a circular transcription substrate), or in some cases, for linearizing a circular transcription substrate, using UNG and endo IV as discussed elsewhere herein. However, the invention is not limited to promoter primers comprising DNA nucleotides or

modified DNA nucleotides, and in some cases, a promoter primer can comprise RNA or modified RNA nucleotides or both DNA and RNA nucleotides or modified nucleotides.

[00255] The nucleic acid target-complementary portion of a promoter primer can be complementary to a specific known sequence in the RNA target in a sample, or it can comprise a mixture of all possible or many possible sequences, such as, but not limited to, random hexamer sequences. Random primer sequences can be made by including nucleotide reagents which are complementary to all four canonical bases during the chemical synthesis of each nucleotide position of the mRNA-complementary portion of the promoter primer. In embodiments of the invention using samples containing mRNA target nucleic acids, which are preferred embodiments, the 3'-end of a promoter primer comprises either a specific sequence that is complementary to a known sequence of a specific mRNA or, if the mRNA has a poly(A) tail at its 3'-end, the 3'-end of the promoter primer can comprise an oligo(dT) sequence. In still other embodiments of the invention for mRNA target nucleic acids, the 3'-end of a promoter primer can comprise a random sequence, such as, but not limited to a random hexamer sequence.

[00256] A promoter primer of the invention comprises a transcription promoter in its 5'-portion. In most embodiments, the transcription promoter comprises a sense promoter sequence that is capable of binding an RNA polymerase of the invention. However, those with knowledge in the art will understand that, due to the fact that the transcription promoter is 5'-of the linear first-strand cDNA that is synthesized by reverse transcription of the RNA target nucleic acid using the promoter primer, an RNA polymerase of the invention cannot use the transcription promoter to synthesize RNA complementary to first-strand cDNA that is complementary to the RNA target nucleic acid. However, if the 5'-end of the linear first-strand cDNA is ligated to its 3'-end so as to form circular first-strand cDNA, then an RNA polymerase of the invention can use the transcription promoter to synthesize RNA complementary to first-strand cDNA that is complementary to the RNA target nucleic acid; thus, a circular first-strand cDNA of this embodiment comprises a transcription substrate of the invention. Thus, a preferred promoter primer of these embodiments of the invention is phosphorylated at its 5'-end in order to facilitate ligation of linear first-strand cDNA by a ligase of the invention under ligation conditions. By means of example, but not of limitation, the 5'-end of the promoter primer can be phosphorylated using T4 polynucleotide kinase and ATP under suitable reaction conditions known in the art. In other embodiments, the promoter primer has a type

1 topoisomerase moiety at its 5'-end in order to facilitate topoisomerase-mediated ligation of linear first-strand cDNA under ligation conditions (e.g., U.S. Patent No. 5,766,891, incorporated herein by reference).

[00257] In general, an anti-sense promoter oligo comprises deoxyribonucleotides. Modified nucleotides or modified linkages should be used in an anti-sense promoter oligo only after carefully determining that they do not substantially affect the ability of the anti-sense promoter oligo to complex with a sense promoter sequence or to bind the RNA polymerase or to affect the ability of the RNA polymerase to initiate transcription using the template strand. However, modified nucleotides can be used for a particular purpose. Similarly, modified linkages, such as, but not limited to alpha-thiophosphate sugar linkages that are resistant to certain nucleases can be used for a particular purpose. An anti-sense promoter oligo can be of any length so long as it has sufficient length to comprise an anti-sense promoter sequence that, when annealed to a sense promoter, makes a functional double-stranded promoter that can be used by an RNA polymerase under transcription conditions to make a transcription product. The oligo comprising the anti-sense promoter can comprise additional nucleotides that are 3'-of or 5'-of the anti-sense promoter sequence so long as the additional nucleotides do not bind the intended target sequence or another component of a method of the invention in a manner that is independent of complexing of the anti-sense promoter sequence with the sense promoter sequence of a sense promoter primer, or otherwise negatively affect the results of the method. If modified nucleotides are used in anti-sense promoter oligo, for a purpose, such as, but not limited to for attaching a labeling moiety, it is preferred that the modified nucleotide is in a nucleotide that does not comprise the anti-sense promoter sequence if possible. If an anti-sense promoter oligo is present in a reaction when steps such as primer extension with a DNA polymerase or ligation with a ligase are performed, the anti-sense promoter oligo is designed so that it cannot participate in these reactions. This is accomplished, for example, by synthesizing an anti-sense promoter oligo that has a dideoxynucleotide or another termination nucleotide on its 3'-end so that it can't be primer-extended and that does not have a phosphate group on its 5'-end (which could participate in a ligation reaction).

D. Ligases and Ligation Methods for Circularizing Linear ssDNA

[00258] It will be clear from the above descriptions of methods for obtaining transcription substrates that it is useful in various embodiments to ligate linear ssDNA to obtain circular ssDNA. The invention is not limited to a specific ligase for circularizing a linear ssDNA molecule and different ligases and ligation methods can be used in different embodiments in order to accomplish a particular purpose. In embodiments that use a ligase, the 5'-end of the linear ssDNA that is ligated to obtain a circular ssDNA must have a 5'-phosphate group or the 5'-end must be phosphorylated using a polynucleotide kinase, such as, but not limited to T4 polynucleotide kinase, during the processes of the method of the invention.

[00259] A ligase that catalyzes non-homologous intramolecular ligation, such as, but not limited to ThermoPhage™ RNA Ligase II (Prokaria, Ltd., Reykjavik, Iceland), is a suitable ligase for ligating linear ssDNA to form a circular ssDNA using the reaction conditions of the manufacturer.

[00260] NAD-dependent DNA ligases that are not active on blunt ends, such as, but not limited to Ampligase® Thermostable DNA Ligase (Epicentre Technologies, Madison, Wisconsin, USA), Tth DNA ligase, Tfl DNA ligase, and Tsc DNA Ligase (Prokaria Ltd., Reykjavik, Iceland) can be used to ligate the 5'-phosphate and 3'-hydroxyl termini of DNA ends that are adjacent to one another when annealed to a complementary DNA molecule, and are suitable ligases in embodiments of the invention that use a ligation splint oligo comprising DNA. However, the invention is not limited to the use of a particular ligase and any suitable ligase can be used. For example, T4 DNA ligase can be used in embodiments of the invention that use a ligation splint. Still further, Faruqui discloses in U.S. Patent No. 6,368,801 that T4 RNA ligase can efficiently ligate DNA ends of nucleic acids that are adjacent to each other when hybridized to an RNA strand. Thus, T4 RNA ligase is a suitable ligase of the invention in embodiments in which DNA ends are ligated on a ligation splint oligo comprising RNA or modified RNA, such as, but not limited to modified RNA that contains 2'-F-dCTP and 2'-F-dUTP made using the DuraScribe™ T7 Transcription Kit (Epicentre Technologies, Madison, WI, USA).

[00261] The invention is also not limited to the use of a ligase for covalently joining the 5'-end to the 3'-end of the same or different nucleic acid molecules in the various embodiments of the invention. By way of example, other ligation methods such as, but not limited to, topoisomerase-mediated ligation (e.g., U.S. Patent No. 5,766,891, incorporated herein by reference) can be used.

E. Modes of Performance of the Methods of the Invention

[00262] Depending on the application and its requirements and constraints, the methods of the invention can be performed in a stepwise fashion, with one set of reactions being performed, followed by purification of a reaction product or removal of reagents or inactivation of enzymes or addition of reagents before proceeding to the next set of reactions, or, in other embodiments for other applications, the methods can be performed as a continuous set of multiple reactions in a single reaction mixture. By way of example, but not of limitation, in some embodiments, each of the separate reactions for transcription using promoter-containing first-strand cDNA as a template for transcription can be performed separately. Still by way of example, in some embodiments in which the methods of the invention are used as part of a diagnostic assay, all of the reactions can be carried out in a single reaction mixture and the products of the transcription reaction may be detected, without ever being isolated.

[00263] The invention also comprises parts or subsets of the methods and compositions of the invention. Thus, the invention comprises all of the individual steps of the methods of the invention that are enabled thereby, in addition to the overall methods.

F. Examples of the Scope of Applications of the Invention

[00264] Those with knowledge in the art will understand that the present invention is novel and very broad in scope and provides improvements in methods, processes, compositions and kits related to making transcription products corresponding to a target nucleic acid sequence comprising RNA, including mRNA, or DNA in a biological sample for many applications. These methods are useful for applications such as, but not limited to, making full-length cDNA and cDNA libraries, improving gene expression analysis, and detecting a target sequence. By way of further example, but not of limitation, the present invention comprises improved methods, processes, compositions and kits that improve upon methods and applications to:

1. amplify nucleic acid molecules *in vitro*
2. amplify a DNA sequence *in vitro*
3. amplify a genomic DNA sequence *in vitro*
4. amplify an RNA sequence *in vitro*
5. amplify mRNA
6. amplify rRNA
7. synthesize RNA
8. synthesize modified RNA, such as, but not limited to RNA containing 2'-fluoro-nucleotides

9. synthesize DNA
10. detect the presence of a nucleic acid sequence in a sample
11. detect the presence of a target nucleic acid sequence in a sample that is indicative of the presence of a target organism
12. detect the presence of a target nucleic acid analyte in a sample
13. detect the presence of a DNA sequence in a sample
14. detect the presence of an RNA sequence in a sample
15. detect the presence (or absence at a detectable level) of a gene in a sample
16. detect the presence of a target organism in a sample
17. detect the presence of a virus in a sample
18. detect the presence of a bacterium in a sample
19. detect the presence of a pathogenic organism in a sample
20. detect the presence of a beneficial organism in a sample
21. identify and quantify nucleic acids associated with RNA and DNA binding proteins
22. detect an oncogene
23. detect an anti-oncogene
24. quantify the level of a virus, a microorganism, a gene, an mRNA, an rRNA, a nucleic acid analyte, or any other nucleic acid of whatever type for whatever purpose.
25. use as a probe
26. use as a probe for an array or microarray
27. make dsRNA or modified dsRNA that can be introduced into human, animal or other eukaryotic cells in serum and without use of a transfection agent.
28. synthesize dsRNA or modified dsRNA *in vitro* for use as RNAi
29. synthesize dsRNA or modified dsRNA *in vitro* for use siRNA
30. make RNAi or modified RNAi that silences a gene encoded by a virus or other infectious agent.
31. make siRNAi or modified siRNA that silences a gene encoded by a virus or other infectious agent.
32. make RNAi or modified RNAi that silences a gene encoded by a plant, animal, human, fungal or other eukaryotic host gene that is involved with and/or interacts with a biological molecule encoded by a virus or other infectious agent.
33. make siRNAi or modified siRNA that silences a gene encoded by a plant, animal, human, fungal or other eukaryotic host gene that is involved with and/or interacts with a biological molecule encoded by a virus or other infectious agent.
34. make RNAi or modified RNAi that silences a non-essential disease-susceptibility gene encoded by a plant, animal, human, fungal or other eukaryotic host gene .
35. make siRNAi or modified siRNA that silences a non-essential disease-susceptibility gene encoded by a plant, animal, human, fungal or other eukaryotic host gene.
36. make RNAi or modified RNAi that silences a non-essential gene encoded by a plant, animal, human, fungal or other eukaryotic host gene, wherein the gene silencing results in a beneficial effect.
37. make siRNAi or modified siRNA that silences a non-essential gene encoded by a plant, animal, human, fungal or other eukaryotic host gene, wherein the gene silencing results in a beneficial effect.
38. make RNAi or modified RNAi that silences a non-essential gene encoded by a plant, animal, fungal or other eukaryotic host gene, wherein the gene silencing results in improved yield, production of a biological molecule, flavor, or other commercially beneficial effect, such as, but not limited to, cold-hardiness, salt-tolerance, shortened growing season, or increased efficiency of utilization of a nutrient.

39. make siRNAi or modified siRNA that silences a non-essential gene encoded by a plant, animal, fungal or other eukaryotic host gene, wherein the gene silencing results in improved yield, production of a biological molecule, flavor, or other commercially beneficial effect, such as, but not limited to, cold-hardiness, salt-tolerance, shortened growing season, or increased efficiency of utilization of a nutrient.
40. diagnose the presence and/or level of an infectious organism
41. diagnose a disease
42. detect the presence of a nucleic acid in an environmental sample
43. differentiate, both qualitatively and quantitatively, between which mRNA molecules are present in different types of cells or in the same type of cells under different conditions or in the same or different types of cells under the same or different conditions or in response to specific stimuli or treatments
44. analyze, both qualitatively and quantitatively, gene expression profiles in cells under different defined conditions
45. analyze, both qualitatively and quantitatively, gene expression profiles in different types of cells under the same defined environmental conditions
46. analyze, both qualitatively and quantitatively, gene expression profiles in cells over time
47. analyze, both qualitatively and quantitatively, gene expression in response to specific stimuli
48. make a library or libraries of mRNA molecules and/or cDNA molecules that are present in one type of cell that are not present in another type of cell, or that are present in one type of cell under certain conditions but not under other conditions (i.e., a subtraction library).
49. map and clone sequences corresponding to the 5'-ends of mRNA's, including, but not limited to, those generated from a specific gene by alternative splicing and promoter usage
50. generate improved templates for more accurate rapid amplification of cDNA ends ("RACE") techniques (e.g., see Flouriot et al., Nucleic Acids Res. 27:e8 (I-iv), 1999).
51. make and/or amplify mRNA for *in vitro* or *in vivo* translation, including, but not limited to coupled or step-wise transcription and translation.
52. amplify RNA and/or DNA or modified RNA and/or DNA present in living cells, such as, but not limited to, tumor or cancer cells from a patient for introduction into dendritic cells (e.g., see U.S. Patents Nos. 5,994,126 and 6,475,483 of Steinman et al., incorporated herein by reference) or other cells from the patient in order to boost or increase an *in vivo* response, such as, but not limited to, an immune response in the patient, with the goal of decreasing the size or the number of cells in the tumor or cancer in the patient.
53. make and/or amplify RNA and/or DNA or modified RNA and/or DNA from a patient and or present in a virus or other infectious agent, wherein the RNA and/or DNA is for use as an RNA vaccine and/or a DNA vaccine, respectively.
54. make RNA or modified RNA that can be introduced into human, animal or other eukaryotic cells in serum and without use of a transfection agent.
55. make modified RNA containing 2'-fluoro-2'deoxynucleotides, such as, but not limited to, 2'-F-dCMP and 2'-F-dUTP, in place of the corresponding canonical nucleotides.
56. produce arrays or microarrays of amplified nucleic acids by attaching the amplification products onto a solid substrate.
57. detect a mutation or a mutated form of a target nucleic acid sequence in a sample
58. quantify the amount of a target nucleic acid or target nucleic acids in a sample.

[00265] Modified RNA molecules that contain 2'-F-dCMP and 2'-F-dUTP are resistant to RNase A-type ribonucleases (Sousa et al., U.S. Patent No. 5,849,546), included herein by reference. Capodici et al., (J. Immunology 169:5196-5201, 2002), included herein by reference, showed that 2'-fluoro-containing dsRNA molecules made using the DuraScribe™ T7 Transcription Kit (Epicentre Technologies, Madison, WI, USA) did not require transfection reagents for delivery into cells, even in the presence of serum. Kakiuchi et al. (J. Biol. Chem. 257:1924-1928, 1982), included herein by reference, showed that use of [(2'-F-dI)$_n$. : (2'-F-dC)$_n$. duplexes were 40-100 times less antigenic than [(rI).sub$_n$. : (rC$_n$.] duplexes, and did not induce an interferon response like [(rI)$_n$. : (rC)$_n$.] duplexes.

I. Kits and Compositions for Embodiments of the Invention for Making a Transcription Product Using a Transcription Substrate Comprising a Target Nucleic Acid Sequence in a Sample

[00266] Important compositions of the invention are Sense Promoter Primers. A Sense Promoter Primer can be provided for primer extension of one specific target sequence or a Sense Promoter Primer can be provided for amplifying a multiplicity of target sequences, such as, but not limited to target sequences comprising all mRNA targets in a sample. In the latter case, a Sense Promoter Primer can be provided that comprises an oligo(dT)$_n$ sequence, or an anchored oligo(dT)$_n$X sequence, or a randomized sequence, such as a random hexamer or random octamer sequence. Still further, multiple specific Sense Promoter Primers can be provided in order to permit amplification of multiple different target sequences in the same sample. Also, multiple different Sense Promoter Primers can be provided which encode different sense promoter sequences that are recognized by different RNA polymerase, such as, but not limited to Sense Promoter Primers that encode sense promoters for T7, T3 and SP6 RNAPs.

[00267] Another composition of the invention can be an Anti-Sense Promoter Oligo that is annealed to a complementary sense promoter in order to obtain a circular or linear transcription substrate having a functional double-stranded promoter.

[00268] Still another composition of an anti-sense promoter oligo of the invention can be an oligonucleotide comprising an anti-sense promoter that is immobilized or attached to a solid support. Preferably, the anti-sense promoter oligo comprising the anti-sense promoter is immobilized on the solid support at or near its 5'-end and the anti-sense promoter sequence is at a sufficient distance from the surface of the solid support so that the sense

promoter in a circular sense promoter-containing first-strand cDNA or a linear sense promoter-containing first-strand cDNA can anneal to the anti-sense sequence so as to make a functional immobilized circular or linear transcription substrate, respectively, when the support is incubated with an RNA polymerase that uses the double-stranded promoter to make a transcription product in a reaction medium under suitable transcription conditions. Preferably, the solid support has a chemical composition and structure so that it does not non-specifically bind nucleic acid from a sample or that comprises a composition of the invention, such as, but not limited to a sense promoter primer. Preferably, the solid support has a chemical composition and structure so that it does not non-specifically bind enzymes, co-factors or other substances in reactions comprising methods of the invention. Without limiting the invention, solid supports can comprise dipsticks, membranes, such as nitrocellulose or nylon membranes, beads, chips or slides used for making arrays or microarrays, and the like. Some solid supports and methods for immobilizing or attaching an anti-sense promoter oligo on a surface or solid support, which can be used for the present invention, are disclosed by Marble et al. in U.S. Patent No. 5,700,667 and in references therein, all of which methods are incorporated herein by reference. Other solid supports which can be used for the present invention are also known in the art and can be used. Numerous other methods for attaching a molecule comprising an oligonucleotide to a surface or other substance are known in the art, and any known method for attaching or immobilizing a molecule comprising an anti-sense promoter oligo can be used to make a composition comprising an immobilized anti-sense promoter oligo is included in the present invention. The composition comprising an oligonucleotide comprising an anti-sense promoter that is immobilized on a solid support can also be used to make functional transcription substrates for making anti-sense transcription products by annealing linear sense promoter-containing second-strand cDNA obtained by rolling circle transcription of a circular anti-sense promoter-containing first-strand cDNA.

[00269] A kit of the invention can comprise one or more Sense Promoter Primers, an Anti-Sense Promoter Oligo and instructions for their use in a method of the invention. The Anti-Sense Promoter Oligo can be in solution, or it can comprise an oligonucleotide that is immobilized on a solid support, as discussed above.

[00270] Another kit of the invention can comprise one or more Anti-Sense Promoter Primers and an Anti-Sense Promoter Oligo, along with instructions for their use in a method of the invention, such as a method for making transcription substrates to obtain

anti-sense transcription products. Similarly, the Anti-Sense Promoter Oligo in the kit can be in solution, or it can comprise an oligonucleotide that is immobilized on a solid support, as discussed above.

[00271] Still another kit of the invention can comprise one or more Sense Promoter Primers or one or more Anti-Sense Promoter Primers, an Anti-Sense Promoter Oligo, whether in solution or immobilized on a solid support, and an optimized composition of a ligase enzyme for circularizing linear promoter-containing first-strand cDNA, as well as instructions for use.

[00272] Yet another kit of the invention can comprise one or more Sense Promoter Primers, an Anti-Sense Promoter Oligo, whether in solution or immobilized on a solid support, an optimized composition of a ligase enzyme for circularizing linear promoter-containing first-strand cDNA, and an optimized composition, such as but not limited to a uracil-N-glycosylase enzyme, for linearizing a circular sense promoter-containing first-strand cDNA having a dUMP residue that was introduced using a Sense Promoter Primer having the dUMP residue, as well as appropriate instructions for use.

[00273] Still another kit of the invention can comprise one or more Sense Promoter Primers or one or more Anti-Sense Promoter Primers, an Anti-Sense Promoter Oligo, whether in solution or immobilized on a solid support, an optimized composition of a ligase enzyme for circularizing linear promoter-containing first-strand cDNA, optionally, an optimized composition for linearizing a circular sense promoter-containing first-strand cDNA, and an optimized composition of the RNA polymerase that uses the double-stranded promoter in the transcription substrates obtained to make a transcription products, as well as instructions for use.

[00274] Another kit can comprise all compositions needed, as individual compositions or as a single optimized combined composition or as a small number of compositions to perform a continuous transcription amplification reaction of the invention, such as but not limited to a reaction as shown in the schematic in FIG. 3.

[00275] Other kits can comprise the compositions above, individually or in combination and/or other compositions, such as, but not limited to:

[00276] (a) a suitable DNA polymerase or reverse transcriptase for making a promoter-containing first-strand cDNA using a Sense or Anti-Sense Promoter Primer;

[00277] (b) a suitable strand-displacing DNA polymerase, such as, but not limited to IsoTherm™ DNA Polymerase (EPICENTRE Technologies, Madison, WI), for rolling circle replication of a circular anti-sense promoter-containing first-strand cDNA;

[00278] Enzymes can be provided in a kit separately or combined into a single ready-to-use solution containing the optimal ratio of each enzyme. A kit comprising enzymes that are used in a method that uses a Promoter Primer can be provided with the Promoter Primer or without a Promoter Primer for customers who wish to prepare their own Promoter Primers for a specific target sequence.

[00279] A kit can be for a specific method, such as, but not limited to, a kit for making a transcription product corresponding to mRNA from a particular type of cell or different cells, whether under the same conditions or different environmental conditions, for gene expression profiling, including generation of sense or anti-sense probes for microarrays, a kit for amplification of an RNA or DNA sequence, including a kit for performing multiple rounds of transcription of a target nucleic acid sequence in a single reaction mixture, a kit for making a transcription product corresponding to a particular target nucleic acid sequence, if present in a sample, that is diagnostic or indicative of a pathogen, a disease gene, a mutated allele, or the like, a kit for an analyte-specific assay, wherein the analyte is a nucleic acid, a kit for making RNAi, including siRNA, or modified RNAi or siRNA, including, but not limited to, 2'-F-dCMP- and 2'-F-dUMP-containing RNAi or siRNA using a DuraScribe™ T7 Transcription Kit (EPICENTRE Technologies, Madison, WI), or any of a broad range of kits that will be understood by those with knowledge in the art by a reading of the description of the invention herein.

[00280] In general, a kit of the invention will also comprise a description of the components of the kit and instructions for their use in a particular process or method or methods of the invention. In general, a kit of the present invention will also comprise other components, such as, but not limited to, buffers, ribonucleotides and/or deoxynucleotides, including modified nucleotides in some embodiments, DNA polymerization or reverse transcriptase enhancers, such as, but not limited to betaine (trimethylglycine), and salts of monvalent or divalent cations, such as but not limited to potassium acetate or chloride and/or magnesium chloride, enzyme substrates and/or cofactors, such as, but not limited to, ATP or NAD, and the like which are needed for optimal conditions of one or more reactions or processes of a method or a combination of methods for a particular application. A kit of the invention can comprise a a set of individual reagents for a particular process or

a series of sets of individual reagents for multiple processes of a method that are performed in a stepwise or serial manner, or a kit can comprise a multiple reagents combined into a single reaction mixture or a small number of mixtures of multiple reagents, each of which perform multiple reactions and/or processes in a single tube. In general, the various components of a kit for performing a particular process of a method of the invention or a complete method of the invention will be optimized so that they have appropriate amounts of reagents and conditions to work together in the process and/or method.

[00281] A kit of the invention can also comprise additional components, such as reaction buffers, control substrates, size markers, detection compositions or detection reagents, and the like, all of which can be provided in quantities to match the need for each component for the proscribed number of intended reactions, but a kit need not contain these components. Still further, a kit can optionally contain detailed instructions and troubleshooting guides.

[00282] Components of a kit may be provided as solutions or as dried powder(s). When reagents and/or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent, in which case, the solvent may also be provided in another container.

[00283] As used herein in the specification, "a" or "an" may mean one or more. As used herein in the claim(s), when used in conjunction with the word "comprising," the words "a" or "an" may mean one or more than one. As used herein "another" may mean at least a second or more.

IX. Signaling System of the Invention

[00284] With respect to this aspect of the invention, a "signaling system" means and comprises the multitude of substances, compositions and environmental conditions comprising a method for detecting the presence or quantity of an analyte in a sample by detecting a transcript that results from transcription of a oligonucleotide that is operably complexed with an analyte-binding substance.

[00285] Other methods for detecting an analyte using transcription are known in the art, including the methods described by Zhang et al. (Proc. Natl. Acad. Sci. USA 98: 5497-5502, 2001), by Eberwine in PCT Patent Application No. WO 02/14476, and by Hudson et al. in U.S. Patent No. 6,100,024.

[00286] However, the present applicants believe that the methods, compositions and kits presented herein are easier to use and make, and can be more easily used for detecting a wide variety of analytes than those which have been described previously by others.

A. Signal Probes of the Invention

[00287] Figure 7 and Figure 8 show two embodiments of methods of the invention that use Signal Probes as part of a signaling system of the invention. As defined herein, a Signal Probe comprises a sense promoter for an RNA polymerase that recognizes a double-stranded promoter for transcription under transcription conditions, and a single-stranded template that is joined 5'-of the sense promoter. If the Signal Probe is complexed or annealed with an anti-sense promoter sequence that is complementary to the sense promoter sequence, a functional promoter is obtained for a cognate RNA polymerase that permits transcription of the template under transcription conditions. The RNA polymerase can be any RNA polymerase that requires a double-stranded promoter, so long as promoter sequences that comprise a double-stranded promoter are used. That is, a single-stranded pseudopromoter or synthetic promoter that is recognized by the RNA polymerase is not suitable for aspects of the invention that used Signal Probe. A suitable RNA polymerase is a T7-type RNA polymerase, preferably, an RNA polymerase chosen from among T7 RNAP, T3 RNAP and SP6 RNAP, and the promoter is a cognate double-stranded promoter that is recognized by the respective RNAP. A "cognate promoter" for a particular RNA polymerase is a promoter that is recognized by that particular RNA polymerase with specifity. Similarly, a "cognate RNA polymerase" for a particular promoter is one that recognizes the particular promoter with substantially greater specifity than another RNA polymerase that recognizes one or more other promoter sequences, thus permitting transcription of the template that is joined with the promoter with specificity even in the presence of other sequences that are not recognized as a promoter by the particular RNA polymerase. The template thus encodes a "signal sequence" and can, but need not, encode one or more other sequences for a particular purpose. By way of example but not of limitation, the template can also encode one or more transcription termination sequences.

[00288] The invention comprises two kinds of Signal Probes. An RCT Signal Probe, which is used in Figure 7, is defined herein as a circular Signal Probe wherein the

template strand is joined to both the 3'-end and the 5'-end of a sequence that comprises the sense promoter sequence. An RCT Signal Probe can be transcribed by rolling circle transcription (RCT), although, as mentioned above, the template of an RCT Signal Probe can also encode transcription termination sequences. The second kind of Signal Probe of the invention, a LINT Signal Probe, is used in Figure 8 and is so named to refer to "LINear Transcription." A LINT Signal Probe is a linear Signal Probe wherein the sense promoter sequence is 3'-of the template. Although the template of a LINT Signal Probe can also encode one or more other sequences in addition to the signal sequence, unless it is desired to terminate transcription from one sequence to another, a transcription terminator sequence is not needed because "run-off" transcription occurs on the linear template. In general, both RCT and LINT Signal Probes comprise deoxyribonucleotides although other nucleotides, including modified nucleotides that do not affect transcription of the signal sequence can be used for a particular purpose. Similarly, modified linkages, such as, but not limited to alpha-thiophosphate sugar linkages that are resistant to certain nucleases can be used for a particular purpose.

B. Analyte-Binding Substances (ABS) Joined to an Oligonucleotide Comprising an Anti-Sense Promoter

[00289] Another important composition of this aspect of the invention is an analyte-binding substance that is joined to an oligonucleotide comprising a sequence for an anti-sense promoter, wherein the anti-sense promoter comprises one strand of a functional double-stranded promoter. Thus, the anti-sense promoter sequence comprising the oligonucleotide that is joined to the analyte-binding substance can complex or anneal with the sense promoter sequence of the Signal Probe to obtain a functional double-stranded promoter for a cognate RNA polymerase. In addition, the anti-sense promoter sequence provides a binding site for the sense promoter sequence in the Signal Probe, thereby enabling complexing or annealing of the Signal Probe to the analyte-binding substance, which in turn can be immobilized or bound to a surface or solid support.

[00290] The template sequence in the Signal Probe encodes a sequence that, once transcribed by the RNA polymerase under transcription conditions, is detectable using any of a variety of methods known in the art. By way of example, but not of limitation, the transcription product could be detected using one or a multiplicity of molecular beacons

that anneal complementary sequence of the transcription products. Alternatively, the template sequence can encode a substrate for Q-beta replicase, which can replicate the substrate and thereby, further amplify the signal. From these examples, it should be clear that the invention is not limited with respect to the transcription products that can be encoded by the template sequence that is joined 5'-of the sense transcription promoter in the Signal Probe. As discussed herein below an analyte-binding substance can be any of a wide variety of compositions for detecting a wide variety of analytes, and all of these are included in the present invention. In general, the oligonucleotide (or "oligo") that is joined to the ABS comprises deoxyribonucleotides, although other nucleotides, including modified nucleotides that do not affect binding of the sense promoter sequence or transcription of the signal sequence can be used for a particular purpose. Similarly, modified linkages, such as, but not limited to alpha-thiophosphate sugar linkages that are resistant to certain nucleases can be used for a particular purpose. The oligo that is joined to the ABS can be of any length, so long as it has sufficient length to comprise an anti-sense promoter sequence that, when annealed to a Signal Probe of the invention, makes a functional double-stranded promoter that can be used by an RNA polymerase under transcription conditions to make a transcription product. The oligo comprising the anti-sense promoter can comprise additional nucleotides that are 3'-of or 5'-of the anti-sense promoter sequence so long as the additional nucleotides do not bind the Signal Probe or another component of a method or assay of the invention in a manner that is independent of complexing of the anti-sense promoter sequence with the sense promoter sequence of the Signal Probe or another component of a method or assay of the invention, or otherwise negatively affect the results of the method or assay. Any suitable method, whether covalent or non-covalent can be used tojoin the 5'-end or 5'-portion of the oligonucleotide to the ABS or to another chemical that serves as a linker to bind the oligonucleotide to the ABS. By way of example, but without limiting the invention, the oligonucleotide can have a biotin moiety that is joined to an ABS that has an attached streptavidin or avidin moiety. It is important that the method and site of joining the oligonucleotide does not substantially affect the ability of the ABS to bind to the analyte, or to another molecule such as a first antibody that is required for the functioning of a particular assay.

[00291] The invention comprises methods, compositions and kits for using the RNA polymerases of the invention as a signaling system for an analyte of any type,

including analytes such as, but not limited to, antigens, antibodies or other substances, in addition to an analyte that is a target nucleic acid.

C. Methods for Using Signal Probes and ABS-Oligos to Detect an Analyte

[00292] Thus, the invention comprises a method for detecting an analyte in or from a sample, the method comprising:

[00293] 1. obtaining an analyte-binding substance-oligonucleotide ("ABS-oligo"), wherein the ABS-oligo comprises an ABS that is joined to a oligonucleotide comprising a sequence for an anti-sense promoter portion of a double-stranded promoter for an RNA polymerase that recognizes the promoter;

[00294] 2. obtaining a Signal Probe, wherein the Signal Probe comprises a sense promoter that is joined to the 3'-end of a template, wherein the sense promoter is sufficiently complementary to the anti-sense promoter of the ABS-oligo to form a complex that can be used for transcription of the template using an RNA polymerase that binds to the complex;

[00295] 3. contacting an ABS-oligo with a surface to which an analyte is bound if present in a sample under analyte-binding conditions that permit the ABS-oligo to bind the analyte if present on said surface;

[00296] 4. washing the surface under conditions that permit removal of unbound ABS-oligo;

[00297] 5. contacting the surface with a Signal Probe under complexing conditions that permit complexing of the Signal Probe with the ABS-oligo if present on the surface;

[00298] 6. optionally, washing the surface under conditions that permit removal of unbound Signal Probe;

[00299] 7. contacting the surface with an RNA polymerase under conditions that permit transcription of a product encoded by the template using the complex between the ABS-oligo and the Signal Probe;

[00300] 8. detecting a transcription product encoded by the template, if present.

[00301] The Signal Probe used in the above described method can be an RCT Signal Probe or a LINT Signal Probe. The step for washing the surface under conditions that permit removal of unbound Signal Probe is optional based on the particular Signal Probe used and whether any "background" transcription products (i.e., "signal") are

detected using the method with a sample that does not contain the analyte. If a background signal is obtained without the washing step, then the washing step to remove unbound Signal Probe should be performed. Since a double-stranded promoter sequence is generally required for transcription by an RNA polymerase of the present invention, the presence of unbound Signal Probes may not cause background signal, in which case, the washing step is not required. A washing step may not be required for methods that use LINT Signal Probes. However, it is known that some RNA polymerases can synthesize RNA by rolling circle transcription using a template comprising a circular ssDNA molecule that is less than about 150 nucleotides, even if the template does not comprise a promoter sequence (U.S. Patent Nos. 5,714,320; 6,077,668; 6,096,880; and 6,368,802). By way of example, but without limitation, the transcription activity by a T7 RNAP on circular ssDNA templates that lack a promoter sequence is higher on smaller circles, such as circular templates that comprise about 25 to about 50 nucleotides. Therefore, a washing step to remove unbound RCT Signal Probes may be required, particularly with RCT Signal Probes up to about 150 nucleotides, and, particularly, with RCT Signal Probes up to about 50 nucleotides. However, whether or not this washing step is required for a particular assay will depend on many factors, including the level of detection, the amount of analyte in the sample, the template used in the Signal Probe, and the level of acceptable background signal for the particular assay. Therefore, the need for the washing step is determined for each particular method (or "assay") of the invention.

[00302] The invention also comprises additional methods, compositions and kits for amplifying the amount of transcription product obtained from transcription of the complex between a Signal Probe and an ABS-oligo. Thus, one embodiment of the invention comprises a method for amplifying the amount of template-complementary transcription product, the method comprising:

[00303] a. obtaining a transcription product by transcription of the template of a Signal Probe that is complexed with an ABS-oligo;

[00304] b. obtaining a sense promoter primer comprising a 3'-end portion that is complementary to the 3'-end of the transcription product and optionally, a phosphate group or a topoisomerase moiety on its 5-end;

[00305] c. annealing the promoter primer to the transcription product;

[00306] d. primer-extending the promoter primer annealed to the transcription product with an RNA-dependent DNA polymerase under DNA synthesis conditions so as to obtain first-strand cDNA;

[00307] e. optionally, removing the RNA that is annealed to the first-strand cDNA;

[00308] f. ligating the first-strand cDNA, wherein the 5'-end is covalently joined to the 3'-end of the first-strand cDNA so as to obtain circular sense promoter-containing first-strand cDNA;

[00309] g. annealing an anti-sense promoter oligo to the circular sense promoter-containing first-strand cDNA so as to obtain a circular substrate for transcription;

[00310] h. contacting the circular substrate for transcription with an RNA polymerase under transcription conditions so as to obtain additional transcription product; and

[00311] i. obtaining the additional transcription product.

[00312] In another embodiment, the circular sense-promoter first-strand cDNA is linearized to obtain linear sense-promoter-containing first-strand cDNA, and then an anti-sense promoter primer is annealed to the sense promoter sequence therein to obtain a linear substrate for transcription. Then, the linear substrate for transcription is contacted with an RNA polymerase under transcription conditions so as to obtain additional transcription product, and additional transcription product is obtained.

[00313] Although a single-stranded promoter cannot be used to obtain a substrate for transcription that comprises a Signal Probe-ABS-oligo complex, the method described above for amplifying the amount of transcription product obtained from transcription of the complex between a Signal Probe and an ABS-oligo using a sense promoter primer to copy the template can use a sense promoter primer comprising a single-stranded sense promoter that is recognized by an RNA polymerase that can make transcription products using the single-stranded promoter. Thus, a sense promoter primer comprising a single-stranded pseudopromoter or synthetic promoter obtained by a method such as that described by Ohmichi et al. (Proc. Natl. Acad. Sci. USA 99:54-59, 2002) or a single-stranded phage N4 promoter, such as, but not limited to the P2 promoter, can be used in embodiments of the method above for amplifying the amount of transcription product. If a single-stranded promoter is used, the cognate RNA polymerase for the promoter is used for transcription of the circular sense promoter-containing first-strand cDNA or linear sense promoter-

containing first-strand cDNA, which is a substrate for transcription. Thus, in these embodiments which use a single-stranded promoter, an anti-sense promoter oligo is not needed to the obtain a circular or linear substrate for transcription for amplifying the transcription products obtained from transcription of the complex between a Signal Probe and an ABS-oligo.

[00314] Still another method of the invention is to amplify the signal of a transcription product from transcription of the complex between a Signal Probe and an ABS-oligo is to use a template sequence in the Signal Probe that encodes a substrate for a replicase such as, but not limited to Q-beta replicase, and to contact the transcription product from transcription of the complex between a Signal Probe and an ABS-oligo with the replicase under replication conditions, and to obtain replicated transcription product, which is detected.

D. **Detection of Signal Sequences** Encoded by the Template of a Signal Probe

[00315] A transcription product from transcription of the complex between a Signal Probe and an ABS-oligo can be detected by any method known in the art. By way of example, but not of limitation, can comprise a substrate for Q-beta replicase, which is detectable, following replication by the replicase under replication conditions, using an intercalating dye such as, but not limited to ethidium bromide. A transcription product can also comprise a sequence that encodes a protein, such as green fluorescent protein, that is detectable following translation of the signal sequence. Without limitation, it can also comprise a sequence that is detectable by a probe, such as, but not limited to a molecular beacon, as described by Tyagi et al. (U.S. Patents Nos. 5,925,517 and 6,103,476 of Tyagi et al. and 6,461,817 of Alland et al., all of which are incorporated herein by reference).

E. Use of the Signaling System with Different Analytes

[00316] A signaling system of the invention can be used for a broad range of analytes and analyte-binding substances. By way of example, but not of limitation, the analyte can be an antigen and the analyte-binding substance can be antibody, or the analyte can be a nucleic acid and the analyte-binding substance can be another complementary

nucleic acid. As discussed below, a large number of other substances exist for which a specific-binding pair can be found, all of which are within the scope of the invention.

[00317] In order to detect an analyte in a sample, an assay of this aspect of the invention uses an analyte-binding substance that "binds" to the analyte under "binding conditions." The analyte-binding substance, which can also be referred to as an "affinity molecule," an "affinity substance," a "specific binding substance," or a "binding molecule" for the analyte, is in turn detected by making a transcription product using a transcription signaling system that is joined to the analyte-binding substance.

[00318] An "analyte" can be any substance whose presence, concentration or amount in a sample is determined in an assay. By way of example, but not of limitation, an analyte can be a biochemical molecule or a biopolymer or a segment of a biopolymer, such as a protein or peptide, including a glycoprotein or lipoprotein, an enzyme, hormone, receptor, antigen or antibody, nucleic acid (DNA or RNA), polysaccharide, or lipid.

[00319] An analyte-binding substance that is a nucleic acid, polynucleotide, oligonucleotide or a segment of a nucleic acid or polynucleotide, including nucleic acids composed of either DNA or RNA, or both DNA and RNA mononucleosides, including modified DNA or RNA mononucleosides, can also be used according to the invention to detect an analyte that does not comprise nucleic acid. For example, a method termed "SELEX," as described by Gold and Tuerk in U.S. Pat. No. 5,270,163, can be used to select a nucleic acid for use as an analyte-binding substance according to the invention. SELEX permits selection of a nucleic acid molecule that has high affinity for a specific analyte from a large population of nucleic acid molecules, at least a portion of which have a randomized sequence. For example, a population of all possible randomized 25-mer oligonucleotides (i.e., having each of four possible nucleic acid bases at every position) will contain 4^{25} (or 10^{15}) different nucleic acid molecules, each of which has a different three-dimensional structure and different analyte binding properties. SELEX can be used, according to the methods described in U.S. Pat. Nos. 5,270,163; 5,567,588; 5,580,737; 5,587,468; 5,683,867; 5,696,249; 5723,594; 5,773,598; 5,817,785; 5,861,254; 5,958,691; 5,998,142; 6,001,577; 6,013,443; and 6,030,776, incorporated herein by reference, in order to select an analyte-binding nucleic acid with high affinity for a specific analyte that is not a nucleic acid or polynucleotide. Once selected using SELEX, nucleic acid affinity molecules can be made by any of numerous known *in vivo* or *in vitro* techniques, including,

by way of example, but not of limitation, automated nucleic acid synthesis techniques, PCR, or *in vitro* transcription.

[00320] Naturally occurring nucleic acid or polynucleotide sequences that have affinity for other naturally occurring molecules such as, but not limited to, protein molecules, are also known in the art. Examples include, but are not limited to certain nucleic acid sequences such as operators, promoters, origins of replication, sequences recognized by steroid hormone-receptor complexes, restriction endonuclease recognition sequences, ribosomal nucleic acids, and so on, which are known to bind tightly to certain proteins. For example, in two well-known systems, the lac repressor and the bacteriophage lambda repressor each bind to their respective specific nucleic acid sequences called "operators" to block initiation of transcription of their corresponding mRNA molecules. Nucleic acids containing such specific sequences can be used in the invention as analyte-binding substances for the respective proteins or other molecules for which the nucleic acid has affinity. In these cases, the nucleic acid with the specific sequence can be used according to this aspect of the invention as the analyte-binding substance for the respective specific protein, glycoprotein, lipoprotein, small molecule or other analyte that it binds. One of several techniques which are generally called "footprinting" (e.g., see Galas, D. and Schmitz, A, Nucleic Acids Res. 5:3161, 1978) can be used to identify sequences of nucleic acids which bind to a protein. Other methods are also known to those with skill in the art and can be used to identify nucleic acid sequences for use as specific analyte-binding substances for use in the invention.

[00321] A peptide nucleic acid (PNA) or a molecule comprising both a nucleic acid and a PNA can also be used according to the invention as an analyte-binding substance for an analyte that is a nucleic acid or polynucleotide. PNA as an analyte-binding substance of the invention provides tighter binding (and greater binding stability) in assays for a nucleic acid analyte (e.g., see U.S. Pat. No. 5,985,563). Also, since PNA is not naturally occurring, PNA molecules are highly resistant to protease and nuclease activity. Antibodies to PNA/DNA or PNA/RNA complexes can be used in the invention for capture, recognition, detection, identification, or quantitation of nucleic acids in biological samples, via their ability to bind specifically to the respective complexes without binding the individual molecules (U.S. Pat. No. 5,612,458).

[00322] The invention also contemplates that a combinatorial library of randomized peptide nucleic acids prepared by a method such as, but not limited to, the

methods described in U.S. Pat. Nos. 5,539,083; 5,831,014; and 5,864,010, can be used to prepare analyte-binding substances for use in assays for analytes of all types, including analytes that are nucleic acids, proteins, or other analytes, without limit. As is the case for the SELEX method with nucleic acids, randomized peptide or peptide nucleic acid libraries are made to contain molecules with a very large number of different binding affinities for an analyte. After selection of an appropriate affinity molecule for an analyte from a library, the selected affinity molecule can be used in the invention as an analyte-binding substance.

[00323] An analyte-binding substance can also be an oligonucleotide or polynucleotide with a modified backbone that is not an amino acid, such as, but not limited to modified oligonucleotides described in U.S. Pat. Nos. 5,602,240; 6,610,289; 5,696,253; or 6,013,785.

[00324] The invention also contemplates that an analyte-binding substance can be prepared from a combinatorial library of randomized peptides (i.e., comprising at least four naturally-occurring amino acids). One way to prepare the randomized peptide library is to place a randomized DNA sequence, prepared as for SELEX, downstream of a phage T7 RNA polymerase promoter, or a similar promoter, and then use a method such as, but not limited to, coupled transcription-translation, as described in U.S. Pat. Nos. 5,324,637; 5,492,817; or 5,665,563, or stepwise transcription, followed by translation. Alternatively, a randomized DNA sequence, prepared as for SELEX, can be cloned into a site in a DNA vector that, once inserted, encodes a recombinant MDV-1 RNA containing the randomized sequence that is replicatable by Q-beta replicase (e.g., between nucleotides 63 and 64 in MDV-1 (+) RNA; see U.S. Pat. No. 5,620,870). The recombinant MDV-1 DNA containing the randomized DNA sequence is downstream from a T7 RNA polymerase promoter or a similar promoter in the DNA vector. Then, following transcription, the recombinant MDV-1 RNA, containing the randomized sequence can be used to make a randomized peptide library comprising at least four naturally-occurring amino acids by coupled replication-translation as described in U.S. Pat. No. 5,556,769. An analyte-binding substance can be selected from the library by binding peptides in the library to an analyte, separating the unbound peptides, and identifying one or more peptides that is bound to analyte by means known in the art. Alternatively, high throughput screening methods can be used to screen all individual peptides in the library to identify those which can be used as analyte-binding substances. Although the identification of an analyte-binding peptide by these methods is difficult and tedious, the methods in the art are improving for doing so, and the expenditure

of time and effort required may be warranted for identifying analyte-binding substances for use in assays of the invention that will be used routinely in large numbers.

[00325] A variety of other analyte-binding substances can also be used in methods for this aspect of the invention.

[00326] For an antigen analyte (which itself may be an antibody), antibodies, including monoclonal antibodies, are available as analyte-binding substances. For certain antibody analytes in samples which include only one antibody, an antibody binding protein such as *Staphylococcus aureus* Protein A can be employed as an analyte-binding substance.

[00327] For an analyte, such as a glycoprotein or class of glycoproteins, or a polysaccharide or class of polysaccharides, which is distinguished from other substances in a sample by having a carbohydrate moiety which is bound specifically by a lectin, a suitable analyte-binding substance is the lectin.

[00328] For an analyte which is a hormone, a receptor for the hormone can be employed as an analyte-binding substance. Conversely, for an analyte which is a receptor for a hormone, the hormone can be employed as the analyte-binding substance.

[00329] For an analyte which is an enzyme, an inhibitor of the enzyme can be employed as an analyte-binding substance. For an analyte which is an inhibitor of an enzyme, the enzyme can be employed as the analyte-binding substance.

[00330] Usually, an analyte molecule and an affinity molecule for the analyte molecule are related as a specific "binding pair", i.e., their interaction is only through non-covalent bonds such as hydrogen-bonding, hydrophobic interactions (including stacking of aromatic molecules), van der Waals forces, and salt bridges. Without being bound by theory, it is believed in the art that these kinds of non-covalent bonds result in binding, in part due to complementary shapes or structures of the molecules involved in the binding pair.

[00331] The term "binding" according to this aspect of the invention refers to the interaction between an analyte-binding substance or affinity molecule and an analyte as a result of non-covalent bonds, such as, but not limited to, hydrogen bonds, hydrophobic interactions, van der Waals bonds, and ionic bonds.

[00332] Based on the definition for "binding," and the wide variety of affinity molecules and analytes which can be used in the invention, it is clear that "binding conditions" vary for different specific binding pairs. Those skilled in the art can easily determine conditions whereby, in a sample, binding occurs between affinity molecule and

analyte that may be present. In particular, those skilled in the art can easily determine conditions whereby binding between affinity molecule and analyte, which would be considered in the art to be "specific binding," can be made to occur. As understood in the art, such specificity is usually due to the higher affinity of affinity molecule for analyte than for other substances and components (e.g., vessel walls, solid supports) in a sample. In certain cases, the specificity might also involve, or might be due to, a significantly more rapid association of affinity molecule with analyte than with other substances and components in a sample.

[00333] "Hybridization" is the term used to refer to the process of incubating an affinity molecule comprising a nucleic acid, or a peptide nucleic acid (PNA) molecule, or a covalently linked, joined or attached nucleic acid-PNA molecule with an analyte comprising a nucleic acid under "binding conditions," which are also called "hybridization conditions." The ability of two polymers of nucleic acid containing complementary sequences to find each other and anneal through base pairing interaction is a well-recognized phenomenon. The initial observations of the "hybridization" process by Marmur and Lane (Proc. Nat. Acad. Sci. USA 46:453, 1960) and Doty, et al. (Proc. Nat. Acad. Sci. USA 46:461, 1960) have been followed by the refinement of this process into an essential tool of modern biology. "Hybridization" also refers to the "binding" or "pairing" of complementary nucleic acid bases in a single-stranded nucleic acid, PNA, or linked nucleic acid-PNA affinity molecule with a single-stranded nucleic acid analyte, which occurs according to base pairing rules (e.g., adenine pairs with thymine or uracil and guanine pairs with cytosine). Those with skill in the art will be able to develop and make conditions which comprise binding conditions or hybridization conditions for particular nucleic acid analytes of an assay. In developing and making binding conditions for particular nucleic acid analytes analyte-binding substances, as well as in developing and making hybridization conditions for particular analytes and capture probes, certain additives can be added in the hybridization solution. By way of example, but not of limitation, dextran sulfate or polyethylene glycol can be added to accelerate the rate of hybridization (e.g., Chapter 9, Sambrook, et al., Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, 1989), or betaine can be added to the hybridization solution to eliminate the dependence of T_m on basepair composition (Rees, W. A., et al., Biochemistry 32:137-144, 1993).

The terms "degree of homology" or "degree of complementarity" are used to refer to the extent or frequency at which the nucleic acid bases on one strand (e.g., of the affinity molecule) are "complementary with" or "able to pair" with the nucleic acid bases on the other strand (e.g., the analyte). Complementarity may be "partial," meaning only some of the nucleic acid bases are matched according to base pairing rules, or complementarity may be "complete" or "total." The length (i.e., the number of nucleic acid bases comprising the nucleic acid and/or PNA affinity molecule and the nucleic acid analyte), and the degree of "homology" or "complementarity" between the affinity molecule and the analyte have significant effects on the efficiency and strength of binding or hybridization when the nucleic acid bases on the affinity molecule are maximally "bound" or "hybridized" to the nucleic acid bases on the analyte. The terms "melting temperature" or "T_m" are used as an indication of the degree of complementarity. The T_m is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands under defined conditions. Based on the assumption that a nucleic acid molecule that is used in hybridization will be approximately completely homologous or complementary to a target polynucleotide, equations have been developed for estimating the T_m for a given single-stranded sequence that is hybridized or "annealed" to a complementary sequence. For example, a common equation used in the art for oligodeoxynucleotides is: T_m =81.5° C + 0.41 (%G+C) when the nucleic acid is in an aqueous solution containing 1 M NaCl (see e.g., Anderson and Young, Quantitative Filter Hybridization, in Nucleic Acid Hybridization, 1985). Other more sophisticated equations available for nucleic acids take nearest neighbor and other structural effects into account for calculation of the T_m. Binding is generally stronger for PNA affinity molecules than for nucleic acid affinity molecules. For example the T_m of 10-mer homothymidine PNA binding to its complementary 10-mer homoadenosine DNA is 73° C, whereas the T_m for the corresponding 10-mer homothymidine DNA to the same complementary 10-mer homoadenosine DNA is only 23° C. Equations for calculating the T_m for a nucleic acid are not appropriate for PNA. Preferably, a T_m that is calculated using an equation in the art, is checked empirically and the hybridization or binding conditions are adjusted by empirically raising or lowering the stringency of hybridization as appropriate for a particular assay. As used herein the term "stringency" is used in reference to the conditions of temperature, ionic strength, and the presence of other compounds, under which nucleic acid hybridizations are conducted. With "high stringency" conditions, nucleic acid base pairing

will occur only between nucleic acid fragments that have a high frequency of complementary base sequences. Thus, conditions of "weak" or "low" stringency are often required when it is desired that nucleic acids that are not completely complementary to one another be hybridized or annealed together.

[00334] With regard to complementarity, it is important for some assays of the invention to determine whether the hybridization represents complete or partial complementarity. For example, where it is desired to detect simply the presence or absence of pathogen DNA (such as from a virus, bacterium, fungi, mycoplasma, protozoan), it is only important that the hybridization method ensures hybridization when the relevant sequence is present. In those embodiments of the invention, conditions can be selected where both partially complementary probes and completely complementary probes will hybridize.

F. Kits and Compositions for Signaling Systems of the Invention

[00335] Important compositions of this aspect of the invention are Signal Probes. Compositions comprising Signal Probes can comprise an RCT Signal Probe or a LINT Signal Probe as described above. Still further, multiple specific Signal Probes, each comprising either a sense promoter for a different RNA polymerase, such as, but not limited to sense a promoter for T7, T3 or SP6 RNAP and/or a different template can be provided in order to detect and/or quantify multiple different analytes in the same sample, or to detect multiple different parts of the same analyte in the sample. By way of example, but without limiting the invention, different Signal Probes can be provided that detect different analyte-binding substances comprising antibodies that recognize antigens comprising different proteins or antigens comprising different antigenic determinants on the same protein. If Signal Probes comprising multiple different templates are used, the templates can encode transcription products that are detectable by multiple different detection molecules, such as, but not limited to multiple different molecular beacons that anneal to different transcription products in order to make a detectable fluorescent signal. Each different molecular beacon can, but need not, have a different fluorescent moiety that is quenched by the quenching moiety, wherein the signal from one fluorescent moiety is distinguishable from the signal from another fluorescent moiety.

[00336] Another important composition of the invention is an analyte-binding substance that is joined to an oligonucleotide comprising a sense promoter sequence (i.e.,

an "ABS-oligo) as described above. Also as discussed elsewhere herein, the analyte-binding substance (ABS) can be any substance that binds the analyte tightly and with specificity so as to obtain specific binding to the analyte in the presence of other substances to which the ABS does not bind.

[00337] A kit can comprise a Signal Probe and an RNA polymerase and other compositions that are used in a method that uses a Signal Probe, but without an ABS-oligo for customers who wish to prepare their own ABS-oligo for a particular analyte.

[00338] A kit of the invention can comprise one or more Signal Probes and an ABS-oligo for detecting an analyte and/or for quantifying an analyte in a sample and instructions for their use in a method of the invention.

[00339] Another kit of the invention can comprise one or more Signal Probes, one or more ABS-oligos for detecting one or more analytes and/or for quantifying one or more analytes in a sample, and an optimized composition of the RNA polymerase that uses the double-stranded promoter in the transcription substrates obtained by complexing a Signal Probe with an ABS-oligo to make a transcription products, as well as instructions for use.

[00340] Still another kit of the invention comprises a kit for for amplifying the amount of template-complementary transcription product obtained from transcription of the complex between a Signal Probe and an ABS-oligo that is bound to an analyte, the kit comprising:

[00341] 1. a sense promoter primer comprising a 3'-end portion that is complementary to the 3'-end of the transcription product encoded by the template of the Signal Probe, and optionally comprising a phosphate group or a topoisomerase moiety on its 5-end;

[00342] 2. optionally, an RNA-dependent DNA polymerase for primer extension of the sense promoter primer using a transcription product encoded by the template of the Signal Probe as a template;

[00343] 3. optionally, a ligase for ligating a first-strand cDNA obtained by primer extension of the sense promoter primer;

[00344] 4. an anti-sense promoter oligo that can complex with the sense promoter of the first-strand cDNA primer extension product so as to obtain a functional double-stranded transcription promoter for an RNA polymerase that binds the promoter and initiates transcription therefrom under transcription conditions;

[00345] 5. optionally, the RNA polymerase that uses the double-stranded promoter;

[00346] 6. optionally, other optimized reaction buffers and compositions for the method, either as individual compositions or as a single optimized combined composition or as a small number of compositions; and

[00347] 7. instructions for their use in a method of the invention.

[00348] Enzymes can be provided in the kit separately or combined into a single ready-to-use solution containing the optimal ratio of each enzyme.

[00349] A kit comprising enzymes and/or other compositions that are used in a method that uses a Signal Probe can be provided with the Signal Probe or without a Signal Probe for customers who wish to prepare their own Signal Probes comprising a different template sequence.

[00350] A kit can be for detecting and/or quantifying a specific analyte, such as, but not limited to, a kit for an analyte-specific assay comprising a pathogen, a disease gene, a mutated allele, or the like.

[00351] In general, a kit of the invention will also comprise a description of the components of the kit and instructions for their use in a particular process or method or methods of the invention. In general, a kit of the present invention will also comprise other components, such as, but not limited to, buffers, ribonucleotides and/or deoxynucleotides, including modified nucleotides in some embodiments, DNA polymerization or reverse transcriptase enhancers, such as, but not limited to betaine (trimethylglycine), and salts of monvalent or divalent cations, such as but not limited to potassium acetate or chloride and/or magnesium chloride, enzyme substrates and/or cofactors, such as, but not limited to, ATP or NAD, and the like which are needed for optimal conditions of one or more reactions or processes of a method or a combination of methods for a particular application. A kit of the invention can comprise a a set of individual reagents for a particular process or a series of sets of individual reagents for multiple processes of a method that are performed in a stepwise or serial manner, or a kit can comprise a multiple reagents combined into a single reaction mixture or a small number of mixtures of multiple reagents, each of which perform multiple reactions and/or processes in a single tube. In general, the various components of a kit for performing a particular process of a method of the invention or a complete method of the invention will be optimized so that they have appropriate amounts of reagents and conditions to work together in the process and/or method.

[00352] A kit of the invention can also comprise additional components, such as reaction buffers, control substrates, size markers, detection compositions or detection reagents, and the like, all of which can be provided in quantities to match the need for each component for the proscribed number of intended reactions, but a kit need not contain these components. Still further, a kit can optionally contain detailed instructions and troubleshooting guides.

[00353] Components of a kit may be provided as solutions or as dried powder(s). When reagents and/or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent, in which case, the solvent may also be provided in another container.

[00354] A broad range of other kits included within the invention will be understood by those with knowledge in the art by a reading of the description of the invention herein.

[00355] As used herein in the specification, "a" or "an" may mean one or more. As used herein in the claim(s), when used in conjunction with the word "comprising," the words "a" or "an" may mean one or more than one. As used herein "another" may mean at least a second or more.